Composition of Lipopolysaccharide from *Pseudomonas syringae* pv. *morsprunorum* and its Digestion by Bacteriophage A7

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Purified LPS from a virulent cherry isolate of *Pseudomonas syringae* pv. *morsprunorum* was a mixture of smooth and rough molecular species. Mild acid hydrolysis yielded a precipitate of lipid A and a carbohydrate fraction which, by gel permeation chromatography, yielded three peaks of material. The first (high molecular weight) peak was composed almost entirely of a rhamnan, the sidechain polysaccharide. The second peak contained core oligosaccharide and comprised rhamnose, glucose, heptose, 2-keto-3-deoxyoctonate (KDO), phosphate, glucosamine, galactosamine and alanine. The third (low molecular weight) peak contained KDO, phosphate and ethanolamine. Lipid A contained glucosamine, phosphate and the fatty acids 12:0, 3-OH 10:0, 2-OH 12:0 (all ester-linked to glucosamine), and 3-OH 12:0, which was amide-linked. The typing phage A7, which uses LPS as its binding site, was found to possess a rhamnanase which split the sidechains from smooth LPS, releasing them as oligosaccharide.

INTRODUCTION

Pseudomonas syringae pv. *morsprunorum* causes bacterial canker of cherry (*Prunus avium*) and plum (*P. domesticum*) trees in England (Garrett *et al.*, 1966). Isolates from these two sources show a high degree of specificity for their homologous hosts. Crosse & Garrett (1963, 1970) established that cherry isolates belonging to race 1 could be distinguished from plum isolates by their susceptibility to the typing phage A7. Freigoun & Crosse (1975) found however that such differentiation was not applicable to cherry isolates of race 2.

Quirk *et al.* (1976) showed that LPS of the race 1 cherry isolate C28 was the receptor of phage A7 by demonstrating inactivation of the phage in the presence of low concentrations of purified LPS. A partial analysis of the material revealed lipid, phosphorus, rhamnose, glucose, ninhydrin-reactive material and 2-keto-3-deoxyoctonate (KDO) but not heptose. In the present paper we describe more fully the structure of LPS from isolate C28, and present evidence to show that phage A7 possesses a rhamnanase (EC 3.2.1.-) that hydrolyses the sidechains of the LPS, releasing them as oligosaccharide. A preliminary report of this work was given earlier (Smith & Hignett, 1981).

METHODS

Organisms. These were provided by Dr C. M. E. Garrett (East Malling Research Station). *Pseudomonas syringae* pv. *morsprunorum* strain C28 was maintained freeze-dried and by monthly serial subculture on nutrient glycerol agar (NGA; Hignett, & Quirk, 1979).

Phage A7 was routinely propagated on cells of strain C28 growing in the diffusible fraction of peptone/yeast extract/glycerol broth (PYG; Crosse & Garrett, 1963). The broth (250 ml) in a 1-litre Erlenmeyer flask was

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Abbreviations: KDO, 2-keto-3-deoxyoctonic acid; S-LPS, R-LPS, smooth and rough LPS; PI₅₀, concentration of a receptor required to inactivate 50% of a population of phage particles.

inoculated with an overnight culture of C28 grown on NGA, and incubated with stirring at 20 °C. At mid-exponential phase (10^9 organisms ml^{-1}) phage was added at a m.o.i. of 1.0–2.0. Lysis was usually complete within 6 h of further incubation, whereupon the lysate was shaken with chloroform, decanted and centrifuged at 10000 *g* for 10 min to remove bacterial debris. The phage suspension, containing typically 10^{11} – 10^{12} p.f.u. ml^{-1} , was stored at 4 °C over chloroform.

Phage adsorption and phage sensitivity tests were done using the procedures described by Crosse & Garrett (1963), Garrett *et al.* (1974) and Quirk *et al.* (1976).

Chemicals, enzymes and authentic standards. Rough LPS from *Escherichia coli* ATCC 12408 (Knox *et al.*, 1967) was a gift from Dr Elizabeth Work. Rough LPS from *Salmonella minnesota* MR595, a Re-mutant, (Lüderitz *et al.*, 1966) was provided by Dr M. Nurminen (National Public Health Institute, Helsinki). Pronase (B Grade), was purchased from the Calbiochem-Behring Corp. Ribonuclease I (four times recrystallized; from bovine pancreas) and protein standards calibration mixture for PAGE (mol. wt range 12300–78000) were obtained from BDH. Ammonium 2-keto-3-deoxyoctonate (KDO), D-mannoheptulose, methyl nonadecanoate and methyl dodecanoate were supplied by Sigma. Authentic bacterial fatty acid methyl ester mixture was purchased from Supelco S A, Crans, Switzerland; 2- and 3-hydroxydodecanoic acids were gifts from Dr Pauline Meadow, University College, London; 3-hydroxydecanoic acid was a gift from Dr S. G. Wilkinson, University of Hull.

Batch culture of bacteria and extraction of LPS. The mineral salts medium, pH 7.0, of Erikson (1945) was modified to contain (l^{-1}): $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 4.3 g; KH_2PO_4 , 1.7 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; trace elements solution (Bauchop & Elsdon, 1960), 1 ml. Glucose (5 g) and polypropylene glycol 2000 (5 μl) were combined with the salts base after being autoclaved separately. Medium (1 litre) was inoculated with the cells from a 24 h old NGA slope of strain C28 and incubated at 18 °C for 40 h with stirring. Cells were harvested, washed twice with distilled water and dried by acetone treatment (Quirk *et al.*, 1976) to yield 1.5–2.0 g dried cells l^{-1} . Purity of cultures was routinely checked using the Gram stain and by plating on nutrient sucrose agar (NSA) to detect levan production (Crosse, 1959). Pathogenicity was confirmed by demonstration of the hypersensitive response (Klement, 1963) in leaves of growing tobacco plants (*Nicotiana tabacum* cv. White Burley).

LPS was extracted from the dried cells with hot aqueous phenol (Westphal & Jann, 1965) and purified by repeated centrifugation at 100000 *g* for 2.5 h after successive treatment with ribonuclease (0.1 mg ml^{-1}) for 16 h and 4 °C, and pronase (0.125 mg ml^{-1}) for 2 h at 37 °C and pH 7.4. Block electrophoresis (Quirk *et al.*, 1976) was used to remove residual nucleic acid and lipid was removed by extraction with ether (Quirk *et al.*, 1976). The purified LPS was stored in solution over chloroform at a concentration of approx. 6 mg ml^{-1} .

Acid hydrolysis of LPS. The sidechain and core components were released from lipid A by hydrolysis of LPS (10–20 mg) with 4–8 ml 0.01 M-HCl at 100 °C for 70 min. Lipid A was extracted with two portions of *n*-hexane. The aqueous residue was dried by rotary evaporation to remove HCl, taken up in water and freed of insoluble protein by membrane filtration (Millipore; 0.45 μm porosity). Sidechain and core components were separated on a column (1.9 $\times$ 51.0 cm) of Sephadex G75 or G50 (fine grade; Pharmacia), using 0.05 M-pyridine/acetic acid buffer, pH 5.4 (Fensom & Meadow, 1970) at a flow rate of 0.25 ml min^{-1} . Fractions (2 ml) were analysed for carbohydrate and phosphate (see below). Peak fractions were pooled and concentrated by rotary evaporation.

Monosaccharide was completely released by heating LPS (2.5 mg ml^{-1}) with 2 M-HCl for 20 min at 100 °C (R. C. Hignett & A. V. Quirk, unpublished observations). Monosaccharide was released from core or sidechain material by the same procedure.

LPS, or isolated lipid A, were totally hydrolysed with 6.1 M-HCl (2 ml per 2.5 mg sample) at 105 °C for 4 h under N_2 or CO_2 . After dilution with water, liberated fatty acids were extracted with ether, dried and esterified using diazomethane. The aqueous residue was dried to remove HCl as described above prior to further analysis.

Alkaline de-O-acylation of LPS. LPS (10 mg) was de-O-acylated in alkali as described by Chester & Meadow (1975) and chromatographed on a column of Sephadex G200. Fractions were analysed for carbohydrate and KDO (see below).

Methanolysis of LPS. Fatty acids were liberated from lipid A or LPS (1–5 mg) as their methyl esters by the method of Salkinoja-Salonen & Boeck (1978). Methyl nonadecanoate (0.2 mg) was the internal standard.

Ester-bound acids were preferentially converted to their methyl esters by treatment of LPS with sodium methoxide (Rietschel *et al.*, 1972). Amide-linked fatty acids were liberated from the ether-insoluble residue by subsequent treatment with 4 M-KOH for 5 h at 100 °C. After acidification with 1 M-HCl, samples were extracted into ether, washed with water, dried and treated with diazomethane in ether.

Partial hydrolysis of LPS by phage A7. Phage A7 (3.3×10^{12} p.f.u., pelleted from a stock suspension in PYG by centrifugation at 40000 *g* for 1 h, and washed once in water), was mixed with 5 mg LPS in 2 ml water and incubated with mild agitation at 20 °C for 2 h. The digested LPS was fractionated at a flow rate of 0.6 ml min^{-1} on a column (1.6 $\times$ 41.0 cm) of Sepharose 4B (Pharmacia) equilibrated with water. Fractions (4 ml) were analysed for carbohydrate and phosphate. Peak fractions were pooled, reduced in volume and submitted to further analysis as detailed in Results.

Paper chromatography, TLC and electrophoresis. Sugars, aminosugars and amino acids were separated by descending chromatography on Whatman No. 1 paper using solvent A, butan-1-ol/pyridine/water (6:4:3, by vol.);

Quirk *et al.*, 1976), and by paper electrophoresis in 6.3% (v/v) formic acid/13% (v/v) acetic acid, pH 2.0. Samples were run for 90 min at 30 V cm⁻¹. Amino compounds were also separated by two-dimensional TLC on cellulose plates (Whatman type CC41) using solvent B, propan-2-ol/formic acid/water (20:1:5, by vol.) followed by C, *tert*-butanol/butan-2-one/ammonia/water (5:3:1:2, by vol.). Sugars and aminosugars were detected using 4-aminobenzoic acid reagent (Saini, 1966); amino compounds were visualized using ninhydrin (Dawson *et al.*, 1969).

Methyl alkanoates were separated from methyl hydroxyalkanoates by TLC at 37 °C on plates of silica gel G (Kieselgel G; Merck) developed successively with solvent D (chloroform/ethyl acetate; 4:1, v/v) for 7 cm, and E (benzene) for 14 cm (Salkinoja-Salonen & Boeck, 1978). Spots were visualized with ethanolic fluorescein (0.05%, w/v), and were eluted with chloroform/methanol (2:1, v/v).

Electrophoresis of LPS on cellulose acetate was done according to the method of Quirk *et al.* (1976). Electrophoresis of LPS on polyacrylamide gel slabs was done by the procedure of Laemmli (1970), and visualized using the silver stain of Tsai & Frasch (1982). In some instances, LPS was visualized by the periodate-Schiff reaction, and protein by staining with Coomassie Brilliant Blue R (Hignett & Quirk, 1979).

Amino compound analysis. The amino compounds obtained from the hydrolysis of approx. 5 mg LPS, lipid A or polysaccharide (see above), were quantified in an automatic analyser (Locarte Co., London, UK), essentially as described by Chester *et al.* (1973), with norleucine (0.5 µmol) as the internal standard.

GC of fatty acid methyl esters. Fatty acid methyl esters were analysed on two columns by flame ionization detection: (i), a Carbowax 20M SCOT splitless glass capillary (0.5 mm × 30 m; Scientific Glass Engineering, UK) operated with temperature programming from 120 °C to 200 °C at 2 °C min⁻¹ and N₂ (2.1 ml min⁻¹) as the carrier gas; (ii), a packed column (3.2 mm × 183 cm) of 3% SP2100-DOH on 100/120 mesh Supelcoport (Supelco) operated with temperature programming from 150 °C to 225 °C at 2 °C min⁻¹ and N₂ (20 ml min⁻¹) as the carrier. Methyl esters of fatty acids were quantified by integration of the areas under peaks, taking the response factor as 1 on a weight basis in all cases (Rietschel *et al.*, 1972), and using methyl nonadecanoate as the internal standard.

Estimations. Protein was determined on intact LPS by the Folin method using bovine serum albumin, fraction V, as the standard. Nucleic acid was determined spectrophotometrically at 260 nm (Dawson *et al.*, 1969). Total amino compounds were quantified with ninhydrin after hydrolysis of the sample in 6.1 M- or 2 M-HCl (Moore & Stein, 1948), with L-alanine as standard. Total aminosugars (hexosamines) were determined colorimetrically (Rondle & Morgan, 1955) after hydrolysis of the sample in 6.1 M- or 2 M-HCl, with D-glucosamine as standard.

Total carbohydrate was determined by the phenol-sulphuric acid reaction (Dubois *et al.*, 1956) against L-rhamnose as standard. Rhamnose and heptose were determined by the method of Osborn (1963), using D-mannoheptulose as the standard for the latter determination. KDO was determined on samples after acid hydrolysis by the method of Weissbach & Hurwitz (1959) as modified by Osborn (1963). Glucose was determined colorimetrically with glucose oxidase (Sigma), after hydrolysis of samples with 2 M-HCl.

Phosphate was determined by the method of Murphy & Riley (1962) after oxidation of samples with 0.05 ml conc. H₂SO₄ containing 1% (w/v) Se and 0.05 ml of 100 vol. H₂O₂ at 200 °C for 1 h.

Calcium and magnesium were determined by atomic absorption spectroscopy, potassium by atomic emission spectroscopy.

RESULTS AND DISCUSSION

Extraction and purity of LPS

Quirk (1976) found that cultures of *P. syringae* pv. *morsprunorum* strain C28 grown on nutrient glycerol broth (NGB) tended to undergo lysis soon after entering stationary phase. In the present investigation this was avoided by growing the organism in modified Erikson's medium. However, LPS prepared from cells grown on NGB had been free of protein, but despite treatment with pronase, LPS extracted from cells grown on salts medium was contaminated with 1–2% (w/w) of tightly associated protein which, in the absence of SDS, migrated on polyacrylamide gels together with the LPS. The absorption spectrum of material after block electrophoresis typically displayed a shoulder at approx. 260 nm ($A_{260}^{1\%} = 0.2–0.3$), indicating the presence of approx. 0.5% (w/w) nucleic acid. Although electrophoresis in SDS on cellulose acetate did not reveal any extra lipid bands, purified LPS also appeared to contain some free long chain fatty acids (see later). Preparations were, however, free of α,ϵ -diaminopimelic acid and thus were not contaminated by peptidoglycan.

The yield of purified LPS was approx. 40 mg (g acetone-dried cells)⁻¹.

Table 1. *Composition of lipopolysaccharide from P. syringae pv. morsprunorum C28*

Assays were performed according to the procedures given in Methods. Fatty acids were determined by weighing the ether-extractable oil from a 6.1 M-HCl hydrolysate of LPS. Lipid A was determined by weighing the hexane-extractable precipitate from LPS hydrolysed in 2 M-HCl for 20 min.

Component	Amount [μmol (mg LPS) $^{-1}$]	
	Analysis 1	Analysis 2
Potassium	0.03	0.004
Calcium	0.31	0.48
Magnesium	0.28	0.20
Phosphorus	1.26	1.38
Rhamnose	1.40	1.90
Glucose	0.20	0.25
Heptose	0.12	0.07
KDO	0.09	0.10
Alanine	0.13	0.19
Glucosamine	ND	0.38
Galactosamine	ND	0.17
Aminosugar phosphates	ND	0.08
Ethanolamine	ND	0.06
Total carbohydrate (rhamnose equivalents)		2.30
Total amino compounds (alanine equivalents)	1.25	1.50
Total amino sugars (glucosamine equivalents)	0.30	0.38
	Amount [μg (mg LPS) $^{-1}$]	
Fatty acids	90	118
Lipid A	200	290
Protein	19	ND

ND, Not determined.

Analysis of whole LPS

Cultures of strain C28 show a pronounced variation in colonial morphology which may be associated with changes in the composition of the LPS. Quirk *et al.* (1976) found that the composition of LPS extracted from different clones of C28 fluctuated widely in respect of the rhamnose:glucose ratio. In our experience too, the composition of LPS varied in this and in other respects, and we encountered one form of the organism from which no LPS could be extracted at all. We have therefore attempted to minimize variation by preparing LPS from cultures made from a single set of lyophilisates. Despite this precaution, differences were still encountered; therefore data are presented from two independent analytical studies.

Hydrolysis of LPS with 2 M-HCl at 100 °C for 20 min gave quantitative release of the lipid A, which amounted to 20% (29% in the second analysis) of the dry weight of the original LPS. The water-soluble residue accounted for 78% (70%) of the original dry weight. In agreement with Quirk *et al.* (1976), paper chromatography of the water-soluble material in solvent A revealed only rhamnose and glucose. In this solvent, heptose migrates with glucose and its presence (see below) was therefore masked.

The aqueous residue from a total acid hydrolysis of LPS was examined by paper electrophoresis, by paper chromatography in solvent A and by TLC on cellulose plates using solvents B and C. Major ninhydrin- and 4-aminobenzoate-positive spots were seen, corresponding in R_F with glucosamine and galactosamine (separable only in solvent A). Two major ninhydrin-positive spots were identified with alanine and ethanolamine; several weaker spots corresponding with other amino acids were also visualized.

Table 1 shows the composition of the LPS analysed in this investigation. The qualitative differences from the earlier findings of Quirk *et al.* (1976) lay in the detection of heptose and protein in these samples. The remaining differences were quantitative. In particular, the amounts of rhamnose and glucose differed, and whereas the maximal rhamnose:glucose ratio was found by Quirk *et al.* to be 4:1, the highest recorded here was 7.6:1. The content of amino

Table 2. *Composition of lipid A from lipopolysaccharide of P. syringae* pv. *morsprunorum* C28

Lipid A was derived from LPS by hydrolysis in 2 M-HCl at 100 °C for 20 min, and extracted quantitatively in *n*-hexane. Analyses were performed according to the procedures given in Methods. Fatty acid methyl esters were analysed by GC using a 0.5 mm × 30 m Carbowax 20M SCOT. Analyses performed on intact LPS gave closely similar results. Glucosamine in analysis 1 was determined by the method of Randle & Morgan (1955) after hydrolysis of lipid A with 6.1 M-HCl for 4 h at 105 °C; in analysis 2 all amino compounds were determined by auto-analysis.

Component	Analysis 1		Analysis 2	
	Amount [μmol (mg LPS) ⁻¹]	Ratios (GlcN = 1)	Amount [μmol (mg LPS) ⁻¹]	Ratios (GlcN = 1)
12:0	0.16	0.99	0.18	1.12
3 HO-10:0	0.15	0.96	0.14	0.88
2 HO-12:0	0.07	0.42	0.18	1.12
3 HO-12:0	0.07	0.46	0.07	0.44
Glucosamine (GlcN)	0.16	1.00	0.16	1.00
Phosphorus	0.18	1.20	ND	ND
Amino-sugar phosphate	ND		0.04	
Galactosamine	ND		0.02	
Alanine	ND		0.02	
Ethanolamine	ND		0.01	

ND, Not determined.

compounds (expressed as alanine equivalents) was found to be approximately fourfold greater in our analysis, probably attributable to the protein contaminant.

Quantitative estimates of the glucosamine, galactosamine, ethanolamine and alanine content were obtained by autoanalysis. In addition, there were small quantities of the full range of protein amino acids, and an unidentified compound, X, which eluted between galactosamine and histidine and which amounted to 0.035 μmol norleucine equivalents (mg LPS)⁻¹. Atomic absorption and emission spectroscopy revealed the presence of approximately equimolar amounts of Ca and Mg, and a trace of K. The fatty acid content was determined gravimetrically on samples of the ether-extractable material from LPS after hydrolysis with 6.1 M-HCl. Its composition is described in the following section.

Composition of lipid A

Lipid A obtained by the hydrolysis of LPS with 2 M-HCl at 100 °C for 20 min contained phosphate and glucosamine, which was identified by autoanalysis and by paper chromatography in solvent A. Lesser quantities of galactosamine and some amino acids were also detected (Table 2).

Fatty acids were liberated from lipid A or from intact LPS as their methyl esters by using methanolic sulphuric acid. Gas chromatography of the transmethylation products revealed four major peaks that co-chromatographed on both Carbowax 20M and SP2100-DOH with methyl dodecanoate, methyl 3-hydroxydodecanoate, methyl 2-hydroxydodecanoate and methyl 3-hydroxydodecanoate, and a minor peak that corresponded with methyl hexadecanoate. Prior separation of methyl dodecanoate from the methyl esters of the hydroxyacids was achieved by TLC on silica gel G plates developed successively in solvents D and E. Treatment of LPS with sodium methoxide released dodecanoic acid, 3-hydroxydodecanoic acid and 2-hydroxydodecanoic acid, which were therefore ester-linked; 3-hydroxydodecanoic acid was released from the partially degraded LPS only by subsequent treatment with 4 M-KOH, and was therefore amide-linked.

Hydrolysis of LPS in 6.1 M-HCl followed by methylation of the ether-extracted free fatty acids using diazomethane gave a relatively greater recovery of methyl hexadecanoate, and two new unidentified peaks emerging close to methyl dodecanoate. Treatment with diazomethane of the ether-extracted oil obtained from transmethylation using methanolic sulphuric acid also gave a significant increase in the amounts recovered of these esters. It would therefore appear that LPS was contaminated with free hexadecanoic acid and two other acids.

Fig. 1. Polyacrylamide gel electrophoresis of SDS-treated LPS from *P. syringae* pv. *morsprunorum* C28. LPS or protein samples were denatured by treatment with SDS according to the procedure of Laemmli (1970) and loaded onto a 12% separating polyacrylamide gel slab (20 cm $\times$ 16 cm $\times$ 1 mm) surmounted by 3% stacking gel. The gel was run for 4 h at 20 mA in 0.025 M-Tris/0.192 M-glycine buffer, pH 8.3, containing 0.1% (v/v) SDS, then fixed and stained with silver as described in Methods. (1) R-LPS of *E. coli* ATCC 12408, 5 μ g; (2) R-LPS of *S. minnesota* MR595, 10 μ g; (3) protein standards mixture (5 μ l) containing proteins of the following molecular weights: 78 000, 66 250, 45 000, 25 700, 17 200, 12 300; (4) LPS of strain C28, 15 μ g. The origins are situated at the top of the photograph.

Glucosamine, phosphate, dodecanoic acid and 3-hydroxydecanoic acid were present in lipid A in approximately equimolar amounts together with half the molar quantity of 3-hydroxydodecanoic acid (Table 2). The quantity of 2-hydroxydodecanoic acid was variable.

This lipid A is therefore similar to that of other pseudomonad species. The four major fatty acids are the same as those found in lipid A from *P. aeruginosa* (Hancock *et al.*, 1970; Wilkinson & Galbraith, 1975), and *P. synchyanea* (Wilkinson *et al.*, 1973). As is usual in pseudomonads, 3-hydroxydodecanoic acid is the amide-linked substituent on glucosamine rather than 3-hydroxytetradecanoic acid, the normal component of enterobacterial LPS.

Hydrolysis of LPS under mildly acidic conditions

Analysis of intact C28 LPS by SDS-PAGE revealed a fast-running component, whose mobility matched that of the rough LPS from *E. coli* ATCC 12408, together with a series of slower-moving bands concentrated towards the origin (Fig. 1). This pattern is consistent with that expected of a mixture of rough and smooth lipopolysaccharides, the latter bearing sidechains of varying length.

Mild acid hydrolysis was used to release intact core and sidechain carbohydrates from the lipid A so as to investigate the molecular structure of the LPS. In preliminary experiments, the release of Lipid A by 1% acetic acid (Fensom & Meadow, 1970) was sometimes incomplete even after 4 h at 100 °C. However, hydrolysis in the presence of 0.01 M-HCl (Chester *et al.*, 1973) was complete in 70 min, and accordingly this procedure was used routinely. After removal of

Fig. 2. Fractionation on Sephadex G75 of carbohydrate obtained from the hydrolysis with 0.01 M-HCl of LPS from *P. syringae* pv. *morsprunorum* C28. LPS (16.5 mg) was hydrolysed with 0.01 M-HCl, and the carbohydrate (12.9 mg) was fractionated on a column of Sephadex G75. Volumes of 0.1 ml were analysed for total carbohydrate (●), and for total phosphate (○). For full experimental details, refer to Methods. Roman numerals I, II, III refer to the peak materials later pooled. Vertical arrow and horizontal bar indicate elution volumes of blue dextran and Cl^- respectively.

insoluble material, the aqueous fraction gave three peaks by gel permeation chromatography on Sephadex G75 (Fig. 2). The first peak (I), emerged with the void volume and consisted of carbohydrate together with very little phosphate. Peak II contained both components. Peak III, which emerged with the totally included volume, contained phosphate but very little carbohydrate. Fractions containing peak material were pooled for further analysis; the results are given in Table 3.

Peak I material gave a coloured product in the phenol-sulphuric acid reaction whose spectrum ($\lambda_{\text{max}} = 480 \text{ nm}$) matched that of authentic rhamnose. Paper chromatography of a 2 M-HCl hydrolysate of peak I material in solvent A revealed only rhamnose. This material also contained 60% of the compound X content of intact LPS, but only very small quantities of phosphate, glucose, KDO, aminosugars and other ninhydrin-reactive compounds, and it is concluded that, like the sidechain fraction of *P. cepacia* (Knirel *et al.*, 1980), it was essentially a simple rhamnan.

Peak II contained rhamnose, glucose and heptose together with glucosamine and galactosamine, amino acids (principally alanine), phosphate, a little KDO and a trace of compound X. Ignoring the last two components, the composition of peak II material approximated to that of a notional octa- or nonasaccharide, $\text{Glc}_2\text{-Rha}_{1-2}\text{-Hep-GlcN}_2\text{-Ala-P}_3$ (where P = phosphate). The core peak was not always well separated from the high molecular weight peak, which may account for the variation seen in the rhamnose:glucose ratio. With the exception of glucosamine, these components are also found in the core of *P. alcaligenes* LPS (Lomax *et al.*, 1974) and of *P. aeruginosa* LPS, the structure of which was recently elucidated (Drewry *et al.*, 1975; Rowe & Meadow, 1983). Peak III contained KDO, phosphate and ethanolamine.

From their molecular weight and residue composition, the three peaks are thought to have represented respectively the complete polysaccharide (sidechain-core), the core, and the link regions of the intact LPS molecule. To confirm this interpretation, LPS was de-O-acylated in ethanolic alkali according to the method of Chester & Meadow (1975) and submitted to chromatography on Sephadex G200. All the applied carbohydrate (as detected by the phenol-sulphuric acid reaction) and KDO eluted together in a single broad peak. In intact LPS the rhamnose is therefore covalently joined to the KDO. However, since SDS-PAGE had indicated that the LPS was a mixture of smooth and rough species, two peaks of KDO-rich material were expected, only one of which should also have contained the rhamnan. Apparently, gel permeation chromatography does not always give a clear separation of the de-O-acylated S- and R-LPS.

Table 3. *Composition of the carbohydrate fractions derived from LPS of P. syringae pv. morsprunorum C28 by hydrolysis with 0.01 M-HCl and separation on Sephadex G75*

Materials from the peaks I, II and III shown in Fig. 2 were analysed according to the procedures given in Methods. Total amino sugars and total amino compounds were estimated after further hydrolysis of the material in 2 M-HCl at 100 °C for 20 min. Individual amino compounds were quantified by autoanalysis after further hydrolysis in 6.1 M-HCl for 4 h at 105 °C under N₂. Samples of LPS (5 mg) were also hydrolysed with 2 M- or 6.1 M-HCl, and the released water-soluble materials were analysed directly according to the procedures given in Methods.

Analysis No.	Dry wt [μg (mg LPS) ⁻¹]	Amount of component [μmol (mg LPS) ⁻¹]													Compound X
		Rhamnose	Glucose	Heptose	KDO	Phosphorus	Total amino sugars	Total amino compounds	GlcN	GalN	Amino- sugar phosphates	Ala	EtN		
0.01 M-HCl hydrolysis	...	1	2	2	1	2	1	2	2	2	2	2	2	2	
Peak no.															
I	280	1.30	1.00	0.04	0.00	0.09	0.01	0.00	0.03	0.02	0.01	0.01	0.01	0.00	
II	250	0.18	0.09	0.16	0.06	0.01	0.01	0.43	0.06	0.06	0.17	0.12	0.13	0.00	
III	350	0.05	0.03	0.05	0.00	0.08	0.06	0.59	0.04	0.04	0.25	0.19	0.01	0.00	
Unresolved mixture	780	1.58	ND	ND	0.09	ND	1.07	ND	0.12	ND	0.40	ND	ND	ND	
2 M-HCl hydrolysis															
Water soluble fraction	ND	1.58	ND	ND	0.08	ND	1.12	1.18	0.14	0.14	0.60	0.58	0.14	0.04	
											0.03	0.10	0.04	ND	

ND, Not determined.

Fig. 3. Elution profile on Sepharose 4B of material derived from LPS of *P. syringae* pv. *morsprunorum* C28 following incubation with phage A7. Phage A7-LPS incubation mixture was fractionated on a column of Sepharose 4B. Volumes of 0.2 ml were taken for total carbohydrate assay (●), and 0.5 ml volumes were assayed for total phosphate (○). For full experimental details, refer to Methods. Letters A and B refer to the peak materials later pooled. Vertical arrow and horizontal bar indicate elution volumes of blue dextran and Cl^- , respectively.

Since it is now apparent that rhamnose is concentrated mainly in the sidechain of this LPS, the fluctuations in the content of this sugar mentioned earlier are probably attributable to variations either in the length of the sidechains or in the ratio of S-LPS to R-LPS.

Interaction of phage A7 with LPS

Quirk *et al.* (1976) demonstrated that purified LPS of strain C28 bound phage A7 and inactivated it efficiently at low concentration ($\text{PI}_{50} \approx 0.05 \mu\text{g ml}^{-1}$ for a suspension containing 10^4 p.f.u. of phage A7 ml^{-1}). In the present investigation, a value for PI_{50} of $0.5 \mu\text{g ml}^{-1}$ was found. By contrast, only weak inactivation ($\text{PI}_{50} = 335 \mu\text{g ml}^{-1}$) was observed with the mixed carbohydrate products resulting from hydrolysis of the LPS with 0.01 M-HCl.

Several other phages that are bound by isolated S-LPS possess a glycosidase located in their tail fibres that catalyses the release of sidechain carbohydrate as oligosaccharide (Lindberg, 1977; Wright *et al.*, 1980). To see whether phage A7 displayed such activity, washed particles (3.3×10^{12} p.f.u.) were gently agitated with LPS (5 mg) in water (2 ml) at room temperature for 2 h. Column chromatography of the product on Sepharose 4B yielded two peaks (Fig. 3). The first, peak A, emerging with the void volume, contained carbohydrate and phosphate, and resembled the parent LPS in giving pronounced light-scatter. The second, peak B, was totally included and contained only carbohydrate.

A sample (1.47 mg) of material from peak A was hydrolysed in 0.01 M-HCl at 100°C . Precipitation of an ether-soluble lipid A was much more rapid than with intact LPS, being complete within 5 min, but heating was continued for 70 min as had been done for the hydrolysis of the parent LPS. After removal of lipid A and HCl, the aqueous residue was applied to a column (1.9×44.0 cm) of Sephadex G50 and eluted with 0.05 M-pyridine/acetic acid buffer, pH 5.4 at 0.25 ml min^{-1} (Fig. 4a). Fractions were collected and analysed for total carbohydrate and phosphate. Nothing was eluted with the void volume, but two peaks emerged (PII and PIII) in the same positions as peaks II and III obtained from the mild acid hydrolysis of the parent LPS. The composition of these peaks was closely similar to that of II and III (Table 4).

A sample (0.40 mg) of the material from peak B when submitted to chromatography on Sephadex G50 emerged in one asymmetrical peak a little ahead of the totally included volume (Fig. 4b). Paper chromatography of the material in solvent A after hydrolysis with 2 M-HCl for 20 min at 100°C revealed the presence of rhamnose as the only sugar. The amount of rhamnose liberated by phage action (Table 4) was the same as the weight of sidechain rhamnose (peak I)

Fig. 4. Elution profiles on Sephadex G50 of carbohydrate fractions derived from LPS of *P. syringae* pv. *morsprunorum* C28 by action of phage A7 rhamnanase. (a) Eluate material of peak A (Fig. 3) was hydrolysed in 0.01 M-HCl and fractionated on Sephadex G50 (3 ml fractions). Volumes of 1 ml were analysed for carbohydrate (●), and for phosphate (○). Designations PII and PIII refer to the materials from acid hydrolysed peak B later pooled. (b) Eluate material of peak B (Fig. 3) was fractionated on Sephadex G50. Volumes of 1 ml were assayed for carbohydrate (▲). For full experimental details, refer to Methods and Results. Vertical arrow and horizontal bars indicate elution volumes of blue dextran and Cl⁻ respectively.

Table 4. *Composition of the carbohydrate fractions derived from LPS of P. syringae* pv. *morsprunorum* C28 by action of phage A7 rhamnanase

The material from peak B (Fig. 3) was analysed directly according to the procedures given in Methods. Peak A material (Fig. 3) was acid-hydrolysed and fractionated on Sephadex G50 to yield the two peaks PII and PIII (Fig. 4a), which were analysed according to the procedures given in Methods. Total amino sugars and total amino compounds were estimated after further hydrolysis of samples in 2M-HCl at 100 °C for 20 min.

	Amount of component [μmol (mg LPS) ⁻¹]				Total amino sugars	Total amino compounds
	Rhamnose	Glucose	Heptose	KDO		
Peak B	1.39	0.00	0.00	0.001	0.01	0.29
0.01 M-HCl hydrolysis products of peak A						
PII	0.23	0.105	0.044	0.005	0.063	0.40
PIII	0.07	0.023	0.000	0.030	0.034	0.31

liberated by mild acid hydrolysis (Table 3). Glucose, KDO, heptose and aminosugar (Elsong-Morgan-positive material) were absent, but some ninhydrin-reactive material was detected (Table 4). Phage A7 therefore possesses a rhamnanase that releases the sidechain from LPS as oligosaccharide, leaving only the core attached to the lipid A. Molar ratios of the residues rhamnose, glucose, heptose, aminosugar and KDO in peak PII material were closely similar to those in peak II of the acid hydrolysate, with the exception that the material derived by phage treatment contained approx. 2–4 times as much rhamnose. Furthermore, the unknown compound X was now detected in the core-derived fraction of the phage digestion product rather than in the sidechain material. In phage-catalysed hydrolysis, the process of sidechain digestion probably halts at some point distal to the location of compound X in the sidechain-core polysaccharide.

The role of LPS from strain C28 as a phage receptor and its partial digestion by a viral enzyme is comparable with several well-documented instances of similar interactions amongst the LPS-binding phages of the *Enterobacteriaceae*. The *Salmonella* phages ε¹⁵, ε³⁴ and P22; the

Escherichia phage Ω 8 and the *Shigella* phage Sf6 all bind to the core or lipid A region of smooth LPS, having removed the O-antigen as oligosaccharide fragments, by the repetitive action of a specific glycosidase borne on the phage tail (Wright *et al.*, 1980).

The site of action and specificity of these phage enzymes has been established. In particular, ϵ^{15} , P22 and Sf6 share the distinction with A7 of possession of rhamnosidases that recognize differing substitution patterns in residues. The rhamnanase of A7 hydrolyses the sidechain of C28 LPS, leaving intact a core region of approximately the same size as that derived from the LPS by acid hydrolysis, though the recovery of a relatively greater amount of rhamnose in the core would be consistent with the enzymic cleavage halting at a point distal to the terminus of unsubstituted core. As with many of the phages of the enterobacteria (Wright *et al.*, 1980), the final binding site for A7 within the residual core-lipid A region is not known and was not found in the carbohydrate liberated by mild acid hydrolysis of intact LPS. The structure of the sidechain, the site of cleavage of the phage A7 rhamnanase and the nature of the oligosaccharide product have been characterized and will be the subject of further communications.

Other *Pseudomonas* phages, for example E79 (Rowe & Meadow, 1983), ϕ PLS1, ϕ PLS44 and ϕ PLS27 (Jarrell & Kropinski, 1981), are known to utilize LPS as their binding site, but there is no evidence that any of these display hydrolytic activity towards O-antigen. Bartell *et al.* (1968) described phage 2 of *P. aeruginosa* BI that released hexosamines from a polysaccharide obtained from bacterial cells. Later work (Castillo & Bartell, 1976) revealed that the phage also bound to isolated lipopolysaccharide with the concomitant release of amino sugars.

The composition and structure of LPS from other virulent and avirulent cherry and plum isolates of *P. syringae* pv. *morsprunorum*, and LPS of the pv. *syringae*, will be described in further communications.

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