EVIDENCE FOR TWO REGIONS IN THE POLYSACCHARIDE MOIETY OF THE LIPOPOLYSACCHARIDE OF *PSEUDOMONAS AERUGINOSA* 8602

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1. Introduction

The lipopolysaccharide components of the walls of the Enterobacteriaceae are composed of a polysaccharide fraction and a lipid fraction (lipid A) which can be readily dissociated by mild acid hydrolysis. The polysaccharide fraction consists of a core region which is common to a number of *Salmonella* and *Escherichia coli*, and a side chain region which differs between strains and is synthesised separately from the core region [1, 2]. Fensom and Gray [3] have recently described the isolation and chemical composition of the lipopolysaccharide of *Pseudomonas aeruginosa* (N.C.T.C. 1999), but attempts to prepare the polysaccharide moiety by mild acid hydrolysis were unsuccessful, and it was not possible to determine whether the pseudomonad polysaccharide had a core and side chain structure, similar to that found in the Enterobacteriaceae.

We have now isolated a lipid-free polysaccharide from the lipopolysaccharide of *P. aeruginosa* 8602 by a modification of the method used for the preparation of 'degraded' Salmonella polysaccharides [4]. Fractionation of the polysaccharide on a column of Sephadex G 50 [5] separated it into two components which we believe correspond to core and side chain regions. We have isolated a mutant of the parent strain which has a defective lipopolysaccharide. Its composition provides further evidence for two regions in the pseudomonad lipopolysaccharide.

2. Materials and methods

Pseudomonas aeruginosa 8602 [6] was maintained on slopes of nutrient agar (Oxoid No. 1). Mutants defective in carbohydrate metabolism were isolated from this strain after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The mutagen (final concentration 100 μg/ml) was added to a suspension of wild-type organisms grown for 4 hr in broth and resuspended in 0.05 M tris-malate buffer (pH 6.0). The suspension was incubated for 30 min at 37°, washed in dilution buffer [6] and used to inoculate fresh cultures. After incubation for 18 hr at 37°, 0.1 ml of suitable dilutions were spread on plates of minimal medium agar [6] with glucose (0.5%, w/v) supplemented with potassium gluconate (0.05% w/v) as carbon source. Mutants defective in glucose metabolism appeared as pin-point colonies after 48 hr incubation. They were picked off and tested for growth in minimal medium containing glucose or gluconate as carbon source. Mutants which grew well on gluconate but slowly on glucose, were grown in 200 ml minimal medium with gluconate as carbon source. The bacteria were harvested after overnight growth and their glucose and rhamnose contents assayed in hydrolysates of freeze-dried bacteria. A mutant designated S-31, proved to contain very little rhamnose and was chosen for further study.

For isolation of lipopolysaccharide, bacteria were grown in minimal medium with citrate (5 g/l) as carbon source, in 5 l flasks at 37° with shaking for 24 hr. They were harvested by centrifugation, washed with dilution buffer and resuspended in the minimum possible volume of distilled water at 4°. After breaking

in the French pressure cell, lipopolysaccharide was isolated from the wet broken cell preparation with 45% phenol as previously described [3] except that the ribonuclease treatment was omitted.

Sugars were identified by descending paper chromatography on Whatman No. 1 paper [3]. Glucose, rhamnose, heptose, KDO, ninhydrin-positive compounds, nucleic acid, and total fatty acids were determined as previously described [3]. Phosphorus was determined by the method of Bartlett [7], total carbohydrate by the phenol-sulphuric acid method [8] and mannose by quantitative paper chromatography [9]. Gas-liquid chromatography was carried out on fatty acids methylated by methanol-sulphuric acid as described by Hancock and Meadow [10] but a diethylene glycol succinate column at 160° was used.

3. Results and discussion

Table 1 shows the composition of the isolated lipopolysaccharides of *P. aeruginosa* 8602 and its mutant S-31. The wild-type lipopolysaccharide is similar to that of strain 1999 but differs in the relative amounts of the sugars and in the absence of fucosamine. The lipopolysaccharide of 8602 contained small amounts of mannose and an unidentified amino compound (X) which runs just after galactosamine in the amino acid analyser and is believed to be an amino sugar. The overall recovery of the polysaccharide was only 71.5% indicating in this strain, as in 1999, the presence of undetected components. The most striking features of composition of the mutant lipopolysaccharide are the virtual absence of rhamnose and the increased amounts of glucose, heptose and total fatty acids. Mannose and unknown X are absent. The fatty acids of the mutant lipopolysaccharide were identical with those previously found in the wild type [11].

The polysaccharide moieties of the wild-type and mutant lipopolysaccharide were prepared by mild acid hydrolysis. Lipopolysaccharide (1% w/v) was suspended in 1% acetic acid in a sealed tube and heated in a boiling water bath until coagulation of lipid A appeared complete. The time required varied from 50–90 min with different preparations. Lipid A was removed from the polysaccharide by centrifugation and filtration through a glass sinter (No. 3 porosity). Determination of total carbohydrate indicated solubilisation of

Table 1

Percentage composition (by weight) of the lipopolysaccharide of *P. aeruginosa* 8602 and mutant S-31. Values for amino acids relate to amino acid residues. All other values relate to the free compounds.

Components	Wild-type	S-31
Rhamnose	8.1	Trace
Glucosamine	7.9	4.7
Mannose	1.0	0
Unknown X[a]	0.4	0
Glucose	5.4	9.5
Heptose[b]	5.4	9.2
Galactosamine	2.2	3.3
Alanine	3.6	1.6
KDO	2.4	4.5
Phosphorus[c]	12.0	17.9
Fatty acids[d]	12.3	25.4
Other amino acids	8.2	1.0
Nucleic acid[e]	2.6	0
Total	71.5	77.1

[a] As glucosamine
[b] As L-*glycero*-D-mannoheptose
[c] As palmitic acid
[d] As H_3PO_4
[e] Estimated as previously described [3].

over 90% of the phenol-sulphuric acid-reacting material of the original lipopolysaccharide. The characteristic fatty acids of the lipopolysaccharide were absent from both polysaccharide preparations indicating complete removal of lipid A.

The isolated polysaccharides were fractionated on a column of Sephadex G 50 [5]. The elution profiles of the two preparations are shown in fig. 1. Two main peaks were obtained from the wild-type preparation. The first of these (I) was eluted immediately after the void volume of the column and was followed by a second peak (II) which appeared to be a doublet. When these two peaks were re-run separately on the same column, both moved in their original positions, but II appeared to split further into two components. No attempt was made to resolve these two components more fully and peak II was subsequently analysed as though it were a single component. In contrast, the mutant preparation yielded mainly peak II with only a trace of peak I. Both preparations yielded a third low

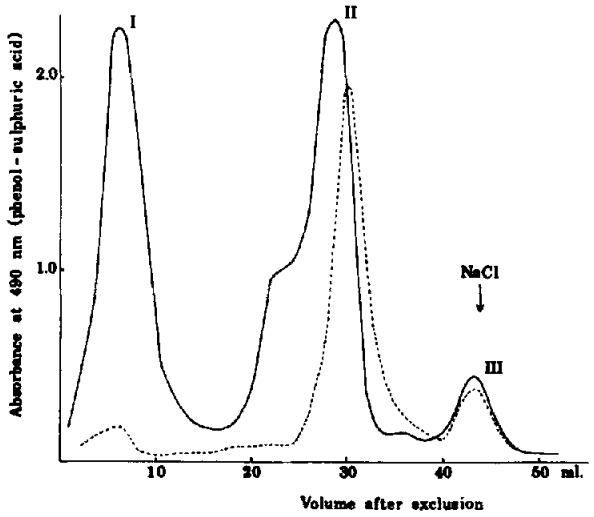

Fig. 1. Fractionation of the degraded polysaccharides from the wild type and mutant lipopolysaccharides on Sephadex G 50. The column (1 X 100 cm) was packed with Sephadex G 50, fine grade and pyridine-acetate (10 ml pyridine + 4 ml glacial acetic acid/l, pH 5.4) was used as effluent. Polysaccharides from 46 mg wild type lipopolysaccharide (——) and from 27 mg mutant lipopolysaccharide (- - - -) were applied.

Table 2

Percentage composition (by weight) of polysaccharide fractions isolated from wild-type and mutant lipopolysaccharide. Values for amino acids relate to amino acid residues. All other values relate to the free compounds.

Component	Wild-type		S-31
	Peak I	Peak II	Peak II
Rhamnose	36.5	8.5	Trace
Glucosamine	8.1	Trace	0
Mannose	1.4	0	0
Unknown X	2.2	Trace	0
Glucose	2.5	14.5	18.1
Heptose	0	8.8	12.3
Galactosamine	0.9	5.6	8.1
Alanine	0.6	2.6	3.6
KDO	0	1.3	1.7
Phosphorus	3.4	18.9	19.6
Total	55.6	60.2	63.4

molecular weight peak (III), analysis of which revealed only KDO and phosphate.

The compositions of fractions I and II are shown in table 2. The wild-type polysaccharide shows a characteristic distribution of components between the two fractions. Rhamnose, glucosamine, mannose and unknown X are concentrated in I while glucose, heptose, galactosamine, phosphate and alanine are concentrated in II. The major component of the mutant polysaccharide (II) is very similar to fraction II from the wild type. There appear to be two distinct regions in the polysaccharide moiety of the wild-type lipopolysaccharide. It is tempting by analogy with the known structure of Salmonella and Escherichia lipopolysaccharides, to regard fractions I and II as side chain and core regions respectively. The mutant would then be similar to the rough mutants of the Enterobacteriaceae in which the side chain region is absent due to a defect in sugar metabolism.

The polysaccharide of the wild-type pseudomonad does not appear to split as clearly into core and side chain regions as does that of *E. coli* [12]. Fraction II contains an appreciable quantity of rhamnose although this is concentrated in Fraction I and absent from the mutant. This could be explained if cleavage of the polysaccharide during acid hydrolysis occurs in such a way that fragments of side chains are left attached to the core. In *E. coli* 'degraded' polysaccharide cleavage occurred mainly within the core to give core fragments left attached to the side chains.

The analytical data do not provide clear evidence for oligosaccharide repeating units in the side-chain region. Rhamnose is the most abundant of the detected components, and the most likely structure would seem to be one in which the other components of I are attached periodically to rhamnose chains. Although the concentrations of glucosamine, mannose and unknown X are low, they appear to be genuine constituents of this region and not of a contaminating polysaccharide, since they are absent in the mutant. However, the low recovery of this fraction, suggesting the possible presence of labile sugars, makes conclusions rather speculative.

The results described show that the polysaccharide component of the lipopolysaccharide of *P. aeruginosa* 8602 is similar to that of the Enterobacteriaceae in consisting of two regions. In the pseudomonad, the main sugars detected in the core region were glucose, heptose and galactosamine. In the Enterobacteriaceae,

glucose and heptose are again major components of the core region but galactose [5] or galactose + glucosamine are also present [1, 5].

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