Isolation of Atypical Lipopolysaccharides from Purified Cell Walls of *Pseudomonas cepacia*

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Wall fragments were prepared from two strains of *Pseudomonas cepacia* and from *P. aeruginosa*, and their contents of readily extractable lipid, pronase-digestible protein and lipopolysaccharide were measured. Lipopolysaccharide extracted from *P. cepacia*, although biologically active, contained no detectable 2-keto-3-deoxyoctonic acid, but contained phosphate, rhamnose, glucose, heptose and hexosamine in concentrations comparable to those found in *P. aeruginosa*.

INTRODUCTION

Pseudomonas cepacia, originally described as a plant pathogen (Ballard *et al.*, 1970), has been implicated in nosocomial infections with increasing frequency (Gelbart *et al.*, 1976; Bassett *et al.*, 1973). The organism is more resistant than other Gram-negative bacteria to quaternary ammonium compounds and most chemotherapeutically useful antimicrobial agents. The relationship between the chemical nature of the cell wall and antimicrobial resistance has long been established (for reviews, see Brown, 1975; Hamilton, 1971). While the walls of *Salmonella typhimurium*, *Escherichia coli* and *Pseudomonas aeruginosa* have been well-characterized, relatively little is known about the walls of *P. cepacia*. In this paper, we describe the composition of the walls of two typically resistant strains of *P. cepacia* and the isolation of an atypical lipopolysaccharide (LPS) from four such strains.

METHODS

Organisms. *Pseudomonas cepacia* 153, originally received from Dr G. L. Gilardi, Hospital for Joint Diseases, New York, N.Y., U.S.A., was maintained in the CIBA-GEIGY culture collection. *Pseudomonas cepacia* ATCC 25416 and *P. aeruginosa* ATCC 9027 were obtained from the American Type Culture Collection. Clinical isolates (*P. cepacia* 2013-75, 1942-75) were obtained from Dr M. Moody, Baltimore Research Center, N.C.I., Baltimore, Md, U.S.A.

Media and growth. Organisms were cultured in a semi-defined glucose/Casamino acids (Difco)/salts medium (Adair *et al.*, 1971) at 30 °C without shaking.

Reagents. Bovine pancreatic ribonuclease A (71 Kunitz units mg⁻¹), 2-thiobarbituric acid and 2-keto-3-deoxyoctonate (KDO) ammonium salt were obtained from Sigma. Glucoheptose (D-glycero-D-guloheptose) was purchased from Research Plus Laboratories, Dover, N.J., U.S.A. Pronase (45 000 proteolytic units g⁻¹) was obtained from Calbiochem. Glucostat (special reagent) was purchased from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. 1-Ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide was purchased from Eastman Organic Chemicals Co., Rochester, N.Y., U.S.A.

Preparation of wall fragments. Batches (20 l) of cells were harvested by centrifugation (7000g) and washed twice with distilled water. Packed cells were stored frozen at -10 °C. They were thawed and suspended in distilled water for breakage by high-frequency sound in a Sonifier Cell Disruptor (model W185D; Heat Systems-Ultrasonics, Plainview, N.Y., U.S.A.). Treatment for 3 min at maximum power, applied as 60 s

bursts with interspersed 1 min cooling intervals, resulted in 100% breakage, as observed by phase contrast microscopy. Broken preparations were centrifuged at 3500g for 10 min; the supernatant was removed and centrifuged at 30000g for 30 min, yielding a pellet of cell wall fragments (CWF) which was washed once with distilled water, resuspended in distilled water and treated with bovine pancreatic RNAase (3 to 5 mg per g wet wt cells before breakage) for 20 h at 37 °C. The RNAase-treated CWF were washed with distilled water, until the absorbance at 260 nm of the supernatant was less than 0.01 on successive centrifugations, and then lyophilized.

Sequential determination of the gross composition of wall fragments. The readily extractable lipid and pronase-digestible protein fractions of CWF were determined by difference after extraction or digestion. Readily extractable lipids were removed by extraction with chloroform/methanol. CWF (100 mg) were stirred at room temperature for 2 h with 15 ml chloroform/methanol (2:1, v/v). The suspension was either carefully filtered or centrifuged for 15 min at 27000g with the supernatant being removed by suction. The residue was re-extracted as before and dried to constant weight. The delipidated material was treated with pronase (Chester & Murray, 1975), and then washed twice with distilled water and dried to constant weight. Lipopolysaccharide was extracted with hot phenol (Westphal & Jann, 1965). All fractions from this procedure were collected and dialysed extensively against running tap water and then distilled water to remove the phenol. After dialysis, the phenol extract was centrifuged at low speed to remove particulate material that had appeared during dialysis. This material was added to the residue fraction. The water and phenol fractions were centrifuged at 105 000g and the pellets were dried to constant weight.

Methods of qualitative analysis. The major sugar components of LPS were identified by means of paper chromatography. LPS (1 to 5 mg) was hydrolysed in 1 M-HCl for 1 h at 100 °C and the hydrolysate was extracted with chloroform to remove lipid components. The partially degraded polysaccharide fraction was further hydrolysed for 4 h in 1 M-HCl at 100 °C. Acid was removed either by ion exchange resin or repeated evaporation *in vacuo*. Descending paper chromatography was performed using Whatman no. 1 paper and the following solvent systems: (A) butan-1-ol/acetic acid/water (4:1:5, by vol.); (B) butan-1-ol/ethanol/water (12:8:4, by vol.) and (C) butan-1-ol/pyridine/water (6:4:3, by vol.). For visualization of the spots, alkaline silver nitrate (Trevelyan *et al.*, 1950), *p*-anisidine (Hough *et al.*, 1950) or ninhydrin were used.

The thin-layer chromatographic method of Buttke & Ingram (1975) was used to identify lipid A in the chloroform fraction. A chloroform extract from hydrolysed *E. coli* LPS was used as a standard.

Methods of quantitative analysis. Pentoses, methyl pentoses and hexoses were measured by the appropriate cysteine-sulphuric acid methods of Dische, as described by Ashwell (1957). Heptose was determined by the Dische cysteine-sulphuric acid method, as modified by Wright & Rebers (1972). D-Glucose was analysed, using glucose oxidase (Glucostat), after hydrolysis with 1 M-HCl for 4 h at 100 °C and subsequent neutralization with a weak base ion-exchange resin. KDO was determined according to Osborn's modification of the method of Weissbach & Hurwitz (Osborn, 1963) and by the newer modification of this method developed by Karkhanis *et al.* (1978). The method of Ghuysen *et al.* (1966) which includes a 4 h hydrolysis with 3 M-HCl was used to determine total hexosamines. A modified version of the Martin-Doty procedure (Heymann *et al.*, 1967) was used for the determination of phosphorus. The carbocyanine dye procedure of Janda & Work (1971) was used to identify and quantify the LPS preparations. Spectra were determined on a Beckman DU spectrophotometer.

Determination of biological activity. Samples of the various fractions extracted according to the Westphal procedure were assayed for pyrogenic activity, according to the standard *United States Pharmacopeia XIX* pyrogen test, using rabbits as the test animal (United States Pharmacopeia Convention Inc., 1974).

RESULTS AND DISCUSSION

Difficulty in extracting LPS from whole bacteria or purified wall fragments of *P. cepacia* led us to adopt the fractionation scheme outlined in Methods. We thus obtained information on the gross composition of the cell wall of these organisms as well as LPS pure enough for chemical analysis.

The wall of *P. cepacia* was grossly similar to that of other Gram-negative organisms of which *P. aeruginosa* is considered representative (Table 1). However, LPS from *P. cepacia* differed in several respects. It distributed into both phases of the Westphal extraction. We believe that the extracted material is LPS because it was pyrogenic in rabbits and reacted with a carbocyanine dye to produce a spectral shift specific to LPS. The composition of the isolated LPSs is shown in Table 2. Compared with LPS from *P. aeruginosa*, that from both strains of *P. cepacia* had less phosphorus and more heptose. Glucose and rhamnose were

Table 1. *Gross composition (% w/w) of cell wall fragments*

Strain	Readily extractable lipid*	Pronase-digestible protein*	LPS			Residue
			Water extract	Phenol extract	Total	
<i>P. aeruginosa</i> ATCC 9027	14.5	50.5	17.8	1.9	19.7	13.2
<i>P. cepacia</i> ATCC 25416	7.72	81.0	1.39	3.99	5.38	4.22
<i>P. cepacia</i> 153	11.4	60.8	4.22	4.35	8.57	8.30

* Determined by difference.

Table 2. *Composition (% w/w) of LPS*

Strain/extract	P	Amino sugar	Heptose	KDO	Glucose	Pentose	Rhamnose	Galactose
<i>P. aeruginosa</i> ATCC 9027 water extract	3.25	12.2	4.05	3.34	8.1	ND	6.4	Tr
<i>P. cepacia</i> ATCC 25416 phenol extract	1.13	5.72	6.4	ND	19.4	Tr*	3.18	ND
<i>P. cepacia</i> 153 water extract	1.19	6.04	16.0	ND	8.0	3.12*	4.25	ND
<i>P. cepacia</i> 153 phenol extract	1.18	5.02	14.0	ND	13.05	0.92*	3.7	ND

ND, Not detectable; Tr, trace (the compound was identified chromatographically but could not be measured).

* Identified chromatographically as xylose.

the major saccharide components of LPS from all organisms. The water and phenol extracts from *P. cepacia* 153 were not strikingly different from each other. KDO was absent from the LPS of the two strains of *P. cepacia* examined in detail. It was also absent from extracts of whole cells of *P. cepacia* 2013-75 and *P. cepacia* 1942-75. Hurlbert & co-workers (1976) have reported that the KDO present in LPS from *Chromatium vinosum* was detected only after a period of hydrolysis five times that needed to liberate the compound from *E. coli*. We have used stronger hydrolytic conditions in our analysis but still found no KDO. In addition, we have found that lengthy hydrolysis with 0.2 M-H₂SO₄ results in KDO destruction. Mild hydrolysis was sufficient to release all the lipid A from *P. cepacia* LPS, as judged by thin-layer chromatography of the chloroform-soluble product. The lipid A was chromatographically similar to that released from *E. coli* under the same conditions.

Although the absence of KDO from LPS is rare, it is not unique. A phenol-soluble, KDO-deficient LPS from *Xanthomonas campestris* has been reported (Hickman & Ashwell, 1966). LPS from *Spirillum serpens*, described by Chester & Murray (1975), also lacks KDO. There are several reports of organisms with LPS that lacks both KDO and heptose (Adams *et al.*, 1970; Hofstad & Kristofferson, 1970; Kaspar, 1976). Our findings in conjunction with these others indicate that the prototypical KDO-lipid A linkage found in *S. typhimurium* (Lüderitz *et al.*, 1973) is not universal.

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