Chemical Characterization of Endotoxic Lipopolysaccharide from Three Strains of *Pseudomonas aeruginosa** (34102)

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Studies on endotoxins from bacterial species outside the *Enterobacteriaceae* are relatively limited but indicate that a chemical similarity may exist among endotoxic lipopolysaccharides (LPS) of all gram-negative bacteria. The principal purpose of this investigation, therefore, was to chemically characterize the endotoxin from *Pseudomonas aeruginosa* in order to compare its composition to endotoxins from other species. A previous study has established similarity of toxic activity of the endotoxin from *P. aeruginosa* when compared to endotoxins of other gram-negative species (1).

Materials and Methods. Three strains of *P. aeruginosa* were used in these studies: American Type Culture Collection (ATCC) strain 10145; Ohio State University (OSU) strain 64; and an animal isolate obtained from the University of Georgia School of Veterinary Medicine, identified here as University of Georgia (UGa) strain VM-1.

Cells were grown in 1-liter Erlenmeyer flasks containing 400 ml of autoclaved medium of the following composition per liter: glucose, 30 g; dehydrated nutrient broth, 8 g; yeast extract, 6 g; Na_2HPO_4, 6 g; KH_2PO_4, 4 g; KNO_3, 10 g; and $MgSO_4 \cdot 7H_2O$, 0.01 g. Cultures were incubated on a rotary shaker at 37° for 6–8 hr or at 30° for 12–14 hr. Cells were harvested by centrifugation and washed three times with distilled water before use. A Waring Blendor was used to free cells of slime during washing and to assure a homogeneous suspension of cells at the time of extraction of endotoxin.

Endotoxin was prepared from either freshly harvested or frozen cells. These were suspended in 0.15 *M* NaCl and extracted with ether according to the method of Foster and Ribi (2). The endotoxin was precipitated with ethanol in the cold and lyophilized.

Hydrolysis was carried out in sealed glass ampoules at 100° for varying periods of time. Both acid and alkaline hydrolyses were found to be effective in releasing bound lipid and in breaking down the polysaccharide portion of the endotoxin. Two hours in 6 *N* HCl appeared to be the most effective hydrolysis for release of monosaccharide constituents. For the liberation of lipid A material, hydrolysis for 1 hr in 1 *N* HCl was found to be effective.

Gas–liquid chromatographic studies were performed in an F and M Model 400 Biomedical Gas Chromatograph equipped with a hydrogen flame ionization detector as previously described (9) but modified as follows: after hydrolysis of the crude endotoxin, the lipid fractions were extracted from the hydrolyzate by shaking with ether:hexane (2:1) and were concentrated at 30° under reduced pressure prior to methylation. Methyl esters of the fatty acids were prepared by esterification with 5% perchloric acid in methanol under reflux at 70° and analyzed in a 6 ft × ¼ in. column of SE-30 Chromosorb W (10%) operated under the following conditions: oven temperature, 200°; injection port and detector temperature, 300°; flow rate, 50 ml/min. A 13 ft × ⅛ in. tetracyanoethylated pentaerythritol on Aeropak 30 (3%) column was also used with the following operating

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conditions: oven temperature, 150°; injection port and detector temperature, 300°; flow rate, 45 ml/min. Identification of methyl esters of fatty acids was made by comparing their relative retention times to the retention times of standard samples and by co-chromatography with standard samples. The semiquantitative data were obtained by the area normalization method.

Thin-layer chromatography of carbohydrates was carried out on 20 × 20-cm glass plates coated with 250-μ layers of silica gel G buffered with boric acid or Kieselguhr G buffered with sodium acetate. 1-Propanol: ethyl acetate:water (7:2:1), *N*-butanol:acetic acid:water (6:3:1), and ethyl acetate:acetic acid:water (3:1:3) were used as solvents. The carbohydrates were visualized with naphthoresorcinol–sulfuric acid, ninhydrin, or thiobarbituric acid spray reagents.

Total carbohydrate for both hydrolyzed and unhydrolyzed LPS material was determined by the anthrone method with glucose as standard (3). Hexosamine was measured by the Boas method (4). Methylpentose was determined with the cysteine–sulfuric acid reaction (5), and heptose by Osborn's modification of the cysteine–sulfuric acid reaction (6). Ketodeoxyoctonate (KDO) was determined using the thiobarbituric acid method of Weissbach and Hurwitz as modified by Osborn (6). Phosphorus was determined after hydrolysis by the method of Fiske and SubbaRow (7). Protein content was measured by the biuret reaction (8). Total lipid was determined indirectly by measuring loss in weight of LPS after hydrolysis and extraction with ether:hexane (2:1).

Experimental results. The overall composition of the endotoxins extracted from the three strains of *P. aeruginosa* is shown in Table I. The composition is expressed as percentage for each of the components of endotoxin from each strain. Assays were not done for *O*-acetyl substituents or for inorganic cations such as Ca^{2+}, Mg^{2+}, Na^{+}, or K^{+}. These results (Table I) show that the compositions of the endotoxic LPS from the three strains of *P. aeruginosa* are similar with regard to the major components and their relative ratio to one another.

TABLE I. Per Cent Composition of Major Components of Endotoxin from Three Strains of *Pseudomonas aeruginosa*.

Component	Strain		
	OSU 64	ATCC 10145	UGA VM-1
	%	%	%
Total carbohydrate	70.2	72.0	67.8
Glucose	12.9	14.2	15.6
Rhamnose	19.3	17.6	20.2
Heptose	8.2	9.5	12.0
Hexosamine[a]	12.3	15.0	14.7
KDO	5.9	6.5	6.3
Lipid	11.9	14.8	12.6
Fatty acids			
Lauric	3.8	1.8	3.2
Palmitic	3.6	4.82	4.0
Stearic	1.2	3.5	2.8
Oleic	0.32	2.2	0.82
Linoleic	0.20	2.13	0.78
Other	1.78	0.35	0.60
Phosphorus	2.75	3.0	2.6
Protein	2.1	2.6	3.0

[a] We did not differentiate between glucosamine and galactosamine in these experiments. In related work, however, it has been found that these two amino sugars, when determined by the method of Stewart-Tull (11), occurred in a molar ratio of 3.73:1 respectively in the lipopolysaccharide material released from isolated cell walls of *P. aeruginosa* strain OSU 64 on exposure to EDTA.

The identification of the carbohydrates in hydrolyzates of the endotoxins as determined by chemical analyses was confirmed by thin-layer chromatography. In addition, ninhydrin-positive spots other than that of hexosamine were observed on thin-layer chromatography but were not positively identified. One of these may have been *O*-phosphorylethanolamine while others may have been amino acids and peptides resulting from partial hydrolysis of the protein which occurred as a contaminant of the endotoxin preparations.

The lipid fractions from the three strains were similar in their major fatty acid compositions. In addition to those shown in Table I, caprylic and capric acids also appeared

to be present in small quantities, but these were difficult to resolve from the solvent peak on the columns used. Two additional fatty acid components in trace amounts were also consistently present but could not be positively identified. The retention times would indicate 14 and 15 carbon chain lengths. These latter fatty acids, as well as capric acid, have been previously shown to be minor components (a) of the readily extractable lipids of the cell wall of *P. aeruginosa* (9) and (b) of the LPS material released from isolated cell walls of this microorganism on exposure to ethylenediaminetetraacetate (EDTA) (10). β-Hydroxymyristic acid, a major component of the endotoxin of enteric bacteria, could not be detected, however, in our preparations of endotoxin from *P. aeruginosa*.

Discussion. It is apparent that there are significant differences in the composition of the endotoxic LPS of *P. aeruginosa* as compared to the LPS of the *Salmonella–Escherichia* group of gram-negative bacteria. The basic structure for the LPS of the latter group of bacteria consists of a core polysaccharide composed of a backbone of heptose, KDO, *O*-phosphorylethanolamine and phosphate to which is attached side chains containing galactose, glucose, and *N*-acetylglucosamine (12, 13). *O*-Specific side chains, composed of galactose, mannose, rhamnose, and a dideoxy sugar, are linked to the basic core structure. Lipid A, attached to the backbone core, is a phosphorylated polyglucosamine acylated mainly with β-hydroxymyristic acid. The backbone core of the LPS of all gram-negative bacteria studied to date appear to be similar in composition and the LPS of *P. aeruginosa* is no exception. The major differences in the LPS of *P. aeruginosa* as compared to those of the enteric bacteria reside in the core side chains and/or *O*-specific side chains and in the lipid A fraction. Excluding the backbone core carbohydrates, no carbohydrates other than glucose, glucosamine, galactosamine, and rhamnose have been detected upon hydrolysis of the LPS of *P. aeruginosa* as reported herein, as reported previously (10), or as reported by other workers (14). Thus, the core side chains and/or *O*-specific side chains of the LPS of *P. aeruginosa* are less complex polysaccharides with respect to the variety of carbohydrates present. In this regard, the LPS of *P. aeruginosa* more closely resembles the LPS of *Neisseria* (15) or of *Xanthomonas* (16) than the LPS of the enteric group of bacteria.

The LPS of *P. aeruginosa* is further distinguished by the absence of β-hydroxymyristic acid as reported herein, as reported previously for related work (9, 10) and as reported by other workers (17). β-Hydroxymyristic acid is considered to be a major component of the lipid A fraction of the LPS of other gram-negative bacteria as exemplified by the enteric bacteria (12, 13). It has been reported by other workers, however, that other β-hydroxy acids are present in the LPS of *P. aeruginosa* (17). While we have failed to confirm this observation, it cannot be excluded that those fatty acids that we were unable to positively identify upon gas–liquid chromatography in these experiments and in related work (9, 10) may be hydroxylated fatty acids.

Summary. A chemical characterization of the endotoxin from three strains of *Pseudomonas aeruginosa* was undertaken to determine the relationships of the endotoxin of this microorganism to endotoxins from other species of gram-negative bacteria.

Hydrolysis of the polysaccharide fraction of the endotoxin of *P. aeruginosa* yielded glucose, glucosamine, galactosamine, rhamnose, heptose, and ketodeoxyoctonate. Fatty acids identified as methyl esters in the lipid fraction were lauric, palmitic, stearic, oleic, and linoleic; caprylic and capric acids also appeared to be present. Two additional minor fatty acid components which could not be positively identified could have been either fatty acids of 14 and 15 carbon chain lengths or hydroxylated fatty acids. β-Hydroxymyristic acid could not be detected.

The endotoxin of *P. aeruginosa* appeared to have a backbone core polysaccharide similar to the *Salmonella-Escherichia* group of gram-negative bacteria. The polysaccharide comprising the core side chains of the endotoxin of *P. aeruginosa* was less complex with respect to the variety of carbohydrates

than that of the enteric bacteria but similar to that of *Neisseria* or *Xanthomonas*. The lipid fraction of the endotoxin of *P. aeruginosa* is further distinguished by the absence of β-hydroxymyristic acid which is a component of the lipid fraction of the endotoxin of other gram-negative bacteria studied to date.

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