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Procedure for Isolation of Bacterial Lipopolysaccharides from Both Smooth and Rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains

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Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. It is now well established that within a single organism, size heterogeneity of this molecule can exist. We have developed a LPS isolation procedure which is effective in extracting both smooth and rough LPS in high yields (51 to 81% of the LPS present in whole cells as quantitated by using hydroxy fatty acid, heptose, and 2-keto-3-deoxyoctonate yields) and with a high degree of purity. The contamination by protein (0.1% by weight of LPS), nucleic acids (1%), lipids (2 to 5%), and other bacterial products was low. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the LPS demonstrated the presence of a high degree of size heterogeneity in the isolated smooth LPS as well as the presence of significant amounts of rough-type LPS. The *Pseudomonas aeruginosa* LPS interacted well with a monoclonal antibody in a variety of immunochemical analyses. The usefulness of the procedure was demonstrated by comparing LPS preparations obtained from wild-type and mutant strains of *P. aeruginosa* and *Salmonella typhimurium*. For example, it was shown that the LPS of an antibiotic supersusceptible mutant Z61 of *P. aeruginosa*, which was previously characterized as identical to wild type with respect to the ratio of smooth to rough LPS molecules isolated by the phenol-water procedure, actually contained only a small proportion of O-antigenic side chains.

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of gram-negative bacteria (29). Some of its functions include a role in the outer membrane permeability barrier (28), resistance to phagocytosis (38, 39), resistance to serum (43), and as a receptor for adsorption of some bacteriophages (37). In addition, its role in bacterial recognition events is becoming recognized (41, 50).

Bacterial LPSs are generally thought to consist of three regions (29, 51), the lipid A, rough core oligosaccharide, and O-antigenic side chain, which are covalently attached to one another. The hydrophobic component, lipid A, generally consists of diglucosamine phosphate with five or six attached fatty acyl chains and is inserted into the outer leaflet of the outer membrane. A significant portion of the fatty acyl chains are often 2- or 3-hydroxy fatty acids, which are unique to LPS in gram-negative bacteria (29). The rough core oligosaccharide consists of about 10 to 12 sugars and contains most of the cellular octose and heptose present as 2-keto-3-deoxyoctonate (KDO) and L-glycero-D-mannoheptose or D-glycero-L-mannoheptose, respectively, as well as a variety of hexoses. The O-antigenic side chain consists of a variable number of repeating saccharide (usually tri- to-pentasaccharide) units and is the portion of the molecule which usually determines serotype specificity (5).

Some bacteria in nature lack this portion of the LPS molecule (12, 18, 47), and mutants which do not contain the O-antigenic side chain are easily obtained by selecting for resistance to certain bacteriophages (10, 16). Bacteria which contain LPS that lacks the O-antigenic side chain often are referred to as rough owing to their colonial morphology, whereas bacteria which have this LPS component are referred to as smooth.

During the last few years, the LPS of several organisms has been shown to be heterogeneous (9, 15, 20, 21, 23, 40). This has led to the proposal that smooth-type organisms may contain some LPS molecules lacking O-antigenic side chains, as well as molecules containing various numbers of covalently bound O-antigenic side-chain units. Indeed, it has been shown that 40 to 50% of the LPS from smooth-type

Escherichia coli O-111 and *Salmonella typhimurium* LT2 contain less than five repeating units of the O-antigenic side chain (9). In addition, the LPS from *Pseudomonas aeruginosa* contains over 80% rough core oligosaccharide without covalently attached O-antigenic side chains (52).

Usually one of two available procedures is used, for the isolation of LPS, depending upon whether the organism displays a rough or smooth phenotype. For smooth-type LPS, the hot phenol-water method developed by Westphal and Jann (49) results in very pure preparations accounting for 1 to 4% of the bacterial cell (dry weight) (24). Rough-type LPS can be obtained by the petroleum ether, chloroform, and phenol method developed by Galanos et al. (8). Although this procedure is far more effective than hot phenol-water for the extraction of this type of LPS, smooth-type LPS is at least partially excluded from the extract (8, 9, 19). Both procedures have been extensively used, and their applicability to a wide variety of strains is well documented. For some smooth bacteria, however, the hot phenol-water procedure has to be modified to obtain a good yield of LPS (17, 20, 43). In addition, some smooth-type LPS molecules do not partition into the aqueous phase (2, 14, 36, 46; A. Kropinski, personal communication).

Studies in our laboratory on the outer membrane of *P. aeruginosa* (1, 20) have indicated that a method of LPS isolation which would extract both smooth and rough forms of the molecule is needed. In this paper, we describe a procedure that extracted both forms of LPS with a high degree of purity in yields that were at least equal to that obtained with either hot phenol-water or petroleum ether, chloroform, and phenol methods. By using this procedure, we have determined that an antibiotic supersusceptible mutant of *P. aeruginosa*, which we previously characterized as identical to wild type with respect to its smooth phenotype, actually contains only a small proportion of O-antigenic side chains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* strains used in this study included wild-type PAO1, originally obtained from A. Kropinski, Queen's University, Kingston, Ontario, Canada; AK 1121, a rough mutant obtained by selection for resistance to phage E79 (16; A. Kropinski, personal communication); Z61, an antibiotic supersusceptible, LPS-altered (20) mutant originally isolated by Zimmermann (54); and the parental strain of Z61, K799. *S. typhimurium* LT2 (SGSC 205) and a rough mutant of this strain (SGSC 206, *rfaH* 481) were obtained from K. Sanderson, *Salmonella* Genetic Stock Center, University of Calgary, Calgary, Alberta, Canada. *E. coli* K-12 (CGSC 6041) was obtained from B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. *P. aeruginosa* strains PAO1, K799 and Z61 were grown in 1% Proteose Peptone no. 2 (Difco Laboratories) at 37°C, whereas strain AK1121 was grown at 30°C in the same medium. *S. typhimurium* and *E. coli* strains were grown in Luria broth (26) at 37°C. All cells were harvested in the midlogarithmic phase, suspended in deionized water, and lyophilized.

LPS analysis techniques. Fatty acid analyses of whole cells and isolated LPS preparations were performed as described previously (20). Assay of KDO was performed by a modification of the method of Osborn et al. (32) with a 15-min hydrolysis period in H_2SO_4. Protein was estimated by the method of Sandermann and Strominger (42). Heptose was determined as described by Wright and Reber (53).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining were performed as described by Tsai and Frasch (47). Immunological procedures including rocket immunoelectrophoresis, Ouchterlony double diffusion, passive hemagglutination, and the enzyme-linked immunoabsorbant assay method were performed as described previously (11).

Method of LPS isolation. We routinely began our isolation procedure with dried bacterial cells to allow quantitation, although a wet cell pellet could also be used in our procedure. Dried bacterial cells (500 mg) were suspended in 15 ml of 10 mM Tris-hydrochloride buffer (pH 8), 2 mM $MgCl_2$, 100 μg of pancreatic DNase I (DN100; Sigma Chemical Co.) per ml, and 25 μg of pancreatic RNase A (R-4875 Sigma) per ml. The cell suspension was then passed twice through a French pressure cell (Amicon Corp.) at 15,000 lb/in^2. To ensure complete cell breakage, the cell lysate was sonicated (Bronwill Scientific Inc.; Bronsonik) for two 30-s bursts at a probe intensity of 75, after which less than 1% of the bacteria remained intact. To ensure efficient nucleic acid digestion, DNase and RNase were added again to final concentrations of 200 and 50 μg/ml, respectively. The suspension was then incubated at 37°C for 2 h. After the incubation period, 5 ml of 0.5 M tetrasodium EDTA (ED4SS; Sigma) dissolved in 10 mM Tris-hydrochloride (pH 8), 2.5 ml of 20% SDS dissolved in 10 mM Tris-hydrochloride (pH 8), and 2.5 ml of 10 mM Tris-hydrochloride (pH 8) were added to give a final volume of the sample of 25 ml containing 0.1 M EDTA, 2% SDS, and 10 mM Tris-hydrochloride at a pH of ca. 9.5. (the purpose of the Tris was to potentiate the effects of EDTA rather than to provide buffering which was provided by the tetrasodium EDTA). The sample was vortexed to ensure solubilization of the components and was then subjected to centrifugation at 50,000 × *g* for 30 min at 20°C to remove peptidoglycan. The supernatant was decanted, and pronase (Sigma) was added to give a final concentration of 200 μg/ml. After this, the sample was incubated overnight at 37°C with constant shaking. After the overnight incubation in the presence of pronase, a precipitate sometimes developed. When this occurred, it was removed by centrifuging the sample in a clinical centrifuge at 1,000 rpm for 10 min. (The supernatant was clear at this point.) Two volumes of 0.375 M $MgCl_2$ in 95% ethanol were added, mixed, and cooled to 0°C (as measured by a thermometer) by placing the flask in a dry ice-ethanol-water (3:2) bath or a −20°C freezer. After the sample had

cooled to 0°C, it was centrifuged at 12,000 × *g* for 15 min at 0 to 4°C. The sample had to be kept as close to 0°C as possible after the precipitate was formed and during the centrifugation. The pellet obtained was suspended in 25 ml of 2% SDS–0.1 M tetrasodium EDTA, dissolved in 10 mM Tris-hydrochloride (pH 8), and sonicated as described above (at this stage the solution was usually clear and the pH ca. 9.5—however, the pH of the solution could be lowered to pH 7 by the dropwise addition of 4 N HCl, to avoid slight saponification of lipids [see comments on the procedure below]). The solution was then incubated at 85°C for 10 to 30 min to ensure the denaturation of SDS-resistant proteins like outer membrane porins (the time and temperature of incubation could be varied depending on the organism; see below). After cooling, the pH was raised (if previously lowered) to 9.5 by the addition of 4 N NaOH. This was found to be necessary for successful protein removal in subsequent steps. Pronase was added to 25 μg/ml, and the sample was incubated overnight at 37°C with constant agitation. After overnight incubation, LPS was precipitated with 2 volumes of 0.375 M $MgCl_2$ in 95% ethanol at 0°C as described above. If the supernatant remained turbid at this time (in our hands this only occurred for smooth *Salmonella* LPS when the solution was not stringently maintained at 0°C during centrifugation), the final concentration of ethanol was increased to 75% by the addition of one extra volume of 0.375 M $MgCl_2$ in 95% ethanol followed by centrifugation at 12,000 × *g* for 15 min at 0 to 4°C.

The pellet after centrifugation was resuspended in 15 ml of 10 mM Tris-hydrochloride (pH 8), sonicated as described previously, and centrifuged in a clinical centrifuge at 1,000 rpm for 5 min to remove insoluble Mg^{2+}-EDTA crystals which sometimes precipitated at this stage. With rough-type organisms, some LPS was occasionally pelleted in this step. This could be recovered by resuspending the pellet (after the supernatant was decanted) and repeating the centrifugation. The supernatant was then centrifuged at 200,000 × *g* for 2 h at 15°C (to prevent precipitation of contaminating SDS) in the presence of 25 mM $MgCl_2$. The pellet which contained the LPS was resuspended in distilled water.

RESULTS

Yields of LPS. To determine the effectiveness of our LPS isolation technique in obtaining both smooth- and rough-type LPS, we isolated LPS from various bacteria displaying these different phenotypes (see Tables 1 and 2). As shown in Table 1, the amount of LPS obtained with our procedure was determined by three different methods. In the first method, the amount of an LPS-specific fatty acid (with *P. aeruginosa*, 2-OH $C_{12:0}$; with *S. typhimurium* and *E. coli*, 3-OH $C_{14:0}$) in the final product was determined and compared with the amount of the equivalent fatty acid present in whole cells. In the second method, we determined the amount of KDO recovered relative to the cell (dry weight). When the yields obtained by these two procedures were compared, after converting the micrograms of KDO to micrograms of LPS according to previously reported KDO (dry weight) percent values for each strain (19, 20, 35, 43), similar recovery values of 60 to 80% were obtained. The third method utilized was to compare the amounts of heptose (an LPS-specific sugar) in the final product with the amount present in whole cells. Since we did not have available a suitable standard for this assay, these values were reported as percent recovered. These determinations also yielded numbers consistent with the hydroxy fatty acid and KDO determinations. We were also able to obtain LPS from several strains of *Agrobacterium tumefaciens*, *Yersinia pestis*, and *Yersinia enterocolitica*, although we did not quantitate the yields.

Purity of LPS preparations. When absorbance scans (A_{300} to A_{200}) of the LPS preparations (0.5 mg/ml) were conducted and the absorbance at 260 and 280 nm was measured, the results indicated that there was less than 0.1% protein and less than 1.0% nucleic acid. In addition, we could detect no protein in our preparations when examined by SDS-PAGE followed by silver staining (see below). Since the silver stain reveals polypeptide bands of 1 ng or less (27), we could conclude that there were no discrete polypeptides at a level of greater than 0.1% contaminating our LPS preparations. When the LPS preparations were examined for the presence of protein by the method of Sandermann and Strominger (42), the results indicated that there was 0.5 to 2.5% protein contamination (microgram of protein per microgram of LPS) depending upon the organism. However, the results obtained with the protein assay may be due to other compounds, including EDTA, hexosamines, and lipids, which are known to interfere with protein determinations in this type of assay (6, 13).

To determine the amount of cellular phospholipids present in the LPS preparations, we determined the amount of $C_{16:1}$ (a fatty acid not found in LPS isolated from *P. aeruginosa* [20], *S. typhimurium*, or *E. coli* [29]) present in the final product and compared this with the amount of this fatty acid present in whole cells. This analysis was performed on all strains and demonstrated that about 2 to 5% of the cellular phospholipids were present in the final product. These could be completely removed from the *P. aeruginosa* LPS preparations, as determined by gas chromatography, by performing either a Folch (7) or a Bligh and Dyer (4) lipid extraction. The fatty acids present in our *P. aeruginosa* LPS preparations were also examined after Folch lipid extractions. A typical experiment resulted in the following mole percentages of fatty acids: 20% 3-OH $C_{10:0}$, 27% $C_{12:0}$, 24% 2-OH $C_{12:0}$, 25% 3-OH $C_{12:0}$, 4% $C_{16:0}$, and 1% others. These

TABLE 1. Yield of LPS obtained by our extraction procedure

Organism	Strain	LPS type	No. of isolations	Yield based on:		
				2-OH fatty acid (% recovery)	Heptose (% recovery)	KDO (μg of KDO per mg of cell [dry wt])
P. aeruginosa	PAO1	Smooth	5	81 ± 20	ND[a]	2.45 ± 0.36
	AK1121	Rough	5	57 ± 10	ND	1.64 ± 0.18
	K799	Smooth	1	ND	65	2.52
	Z61	Semirough (altered)	5	59 ± 6	ND	2.19 ± 0.30
S. typhimurium	SGSC205	Smooth	2	57, 53	51, 72	ND[b]
	SGSC206	Rough	4	65, 57	54 ± 15	1.07, 1.56
E. coli	CGSC6041	Rough	4	75 ± 17	54 ± 17	2.16, 1.98

[a] ND, Not done.
[b] Owing to interference from abequose, a component of *S. typhimurium* O-antigen which causes interference with the KDO assay, accurate estimations of KDO could not be obtained for this strain.

fatty acids have been previously demonstrated to be the major fatty acids in *P. aeruginosa* LPS (20).

The amount of SDS present in the final product was less than 2% by weight of LPS as detected by studies using [^{35}S]SDS (New England Nuclear Corp.), however, this could be reduced to less than 0.1% by resuspending the ultracentrifuge pellet in 10 mM Tris-hydrochloride (pH 8) and subjecting the sample to further centrifugation at 200,000 × *g* as described above. The SDS could be completely removed from the *P. aeruginosa* preparations by performing a Folch lipid extraction (7) without doing the NaCl backwash since some of the SDS which was extracted into the $CHCl_3$-CH_3OH supernatant partitioned with the remaining LPS into the 0.9% NaCl aqueous phase upon washing. In the Folch lipid extractions, some loss of LPS did occur. For example, in strain PAO1, 12% of the

TABLE 2. Comparison of LPS yields by the present method with the procedures of Westphal and Jann (phenol-water) and Galanos et al. (phenol-chloroform-petroleum ether)

Organism	Strain	LPS type	LPS content (% cell [dry wt])	Yields (% cell [dry wt])		
				Present method	Procedure of Westphal and Jann[a]	Procedure of Galanos et al.[a]
P. aeruginosa	PAO1	Smooth	7.3[b]	5.9	0.85 (19)	0 (19)
	AK1121	Rough	6.0[c]	3.4	1 (19)	2.2 (19)
	K799	Smooth	9.1[b]	5.9	1 (20)	
	Z61	Semirough	8.4[b]	4.9	1 (20)[d]	
S. typhimurium	SGSC205	Smooth	8.5[e]	4.7	1–4 (24)	
	SGSC206	Rough	3.5[e]	2.6	1 (8)	1–5 (8)
E. coli	CGSC6041	Rough	3.1[e]	2.3	1 (8)	1–2 (35)

[a] Numbers in parentheses refer to the references from which these yields were obtained.
[b] Calculated from whole cell fatty acid analysis assuming that 3-OH $C_{12:0}$ constituted 5% (by weight) of the LPS (20). Similar numbers were obtained from the KDO values in Table 1 by estimating the KDO was 4.3% of the weight of LPS (20).
[c] Calculated from the KDO values in Table 1 by estimating that KDO was 4.8% of the weight of LPS for this strain (19).
[d] Yields of Z61 LPS cited here were improved by a modification of the procedure of Westphal and Jann (20). The standard procedure yielded only 0.23 ± 0.08% of cell (dry weight) (A. Carey and R. E. W. Hancock, unpublished data).
[e] Calculated from the 3-OH $C_{14:0}$ contents of whole cells by using the published chemical compositions to estimate the % (by weight) of LPS that was 3-OH $C_{14:0}$. Thus, the average molecular weight of *S. typhimurium* SGSC205 LPS was calculated as 12,400 (assuming an average of 9 O-antigen repeating units per molecule of LPS as demonstrated by Palva and Makela [33]), whereas, the two rough LPSs had molecular weights of around 4,300 (43). Three 3-OH $C_{14:0}$ molecules per molecule LPS were assumed (44).

KDO remained soluble and stayed in the $CHCl_3$-CH_3OH phase, and although this could be recovered by a 0.9% NaCl backwash as stated above, some SDS also partitioned with this population of LPS. A Bligh and Dyer (4) two-phase $CHCl_3$-CH_3OH extraction was also effective in removing phospholipids from the LPS preparations, although some SDS remained with the LPS. In addition, when this type of extraction was performed on LPS obtained from strain Z61 but not the other *P. aeruginosa* strains examined, 15% of the KDO partitioned into the chloroform phase. We did not determine the amount of SDS contamination found in *S. typhimurium* or *E. coli* LPS preparations.

Characterization of LPS. LPS obtained by our procedure was subjected to SDS-PAGE and visualized by the sensitive silver staining method of Tsai and Frasch (47). As shown in Fig. 1 and in agreement with our previous results (20), wild-type *P. aeruginosa* LPS displayed some degree of size heterogeneity as evidenced by the various bands located in the middle to upper regions of the gel. In addition, these results demonstrated that a considerable portion of the LPS of wild-type *P. aeruginosa* is rough. When a rough mutant was examined, bands corresponding to the O-antigenic side chain were absent (Fig. 1A, lane 6). We then compared LPS obtained from another wild-type *P. aeruginosa* K799 and a mutant Z61 derived from this strain which is supersusceptible to a wide variety of antibiotics. The results clearly demonstrated that in the mutant strain most of the smooth-type LPS was missing so that the organism appeared rough, despite its smooth colony appearance (20). If, however, gels were overloaded with LPS obtained from strain Z61 (with between 30 to 40 μg of LPS), O-antigenic side chains could be seen (Fig. 1C). This was consistent with the observation that this mutant is sensitive to a variety of smooth-specific phages (1). LPS obtained from *S. typhimurium* LT2 displayed size heterogeneity observed by other authors (9, 33). Although not evident in this reproduction, the bands in the lower portion of the gel consisted of doublets similar to those observed by Goldman and Leive (9). Interestingly, the top band of each doublet stained orange with silver stain, whereas the bottom band stained dark brown. The rough variant of *S. typhimurium* used in this study appeared rough as evidenced by the lack of O-antigen side chains (Fig. 1A, lane 2). However, since this strain was an *rfaH* mutant which has been shown to result in various types of LPS molecules being present (23), we decided to examine this strain for the presence of smooth-type LPS. When 40 μg of LPS was added to the gel, the presence of multiple bands extending from the lower to the upper regions of the gel were evident (Fig. 1B).

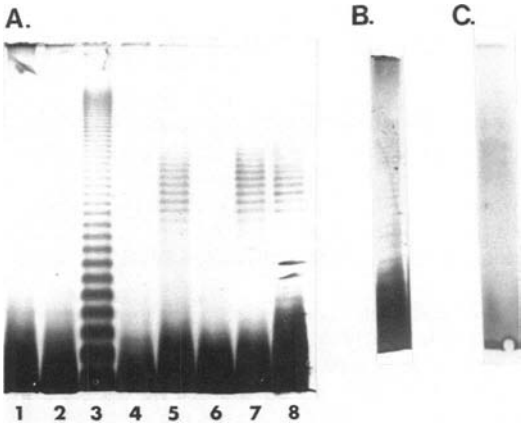

FIG. 1. LPS obtained by our extraction procedure and subjected to SDS-PAGE (15% acrylamide, 4 M urea). Samples were mixed 1:1 with buffer containing 0.1 M Tris-hydrochloride (pH 6.8), 2% SDS (wt/vol), 1% 2-mercaptoethanol (vol/vol), 0.001% bromophenol blue (wt/vol), 10% sucrose, and 40 mM EDTA. The samples were heated at 100°C for 5 min, cooled, and applied to the gel. Gels were stained by the method of Tsai and Frasch (47). (A) Lanes 1 through 3, 5 μg of LPS was added; lanes 4 through 8, 10 μg of LPS was added. LPS was obtained from: lane 1, *E. coli* CGSC 6041; lane 2, *S. typhimurium* SGSC 206; lane 3, *S. typhimurium* SGSC 205; and lanes 4, 5, 6, 7, and 8 are *P. aeruginosa* strains Z61, K799, AK1121, PAO1, and PAO1 isolated by the phenol/water procedure of Westphal and Jann (49), respectively. (B) The same sample as in lane 6 with the exception that 40 μg of LPS was applied to the gel. (C) A sample of mutant Z61 LPS which was overloaded (30 μg) to show the presence of O-antigen side chains; owing to the amount of rough core present, a smearing of the faint O-antigen side-chain bands has occurred.

LPS obtained by our extraction procedure has also been used successfully as an antigen to stimulate antibody production in mice for the generation of monoclonal antibodies with determinants directed against different regions of the LPS molecule, to coat ELISA plates, and to use in rocket immunoelectrophoresis assays, Ouchterlony double diffusion, and passive hemagglutination (11). As determined by quantitative rocket immunodiffusion assays (3), LPS obtained by our procedure demonstrated the same affinity, as LPS obtained by the Westphal and Luderitz procedure, for a monoclonal antibody (MA1–8; reference 11) specific for an O-antigenic determinant.

Comments on the procedure. (i) Large and small scale isolations. The isolation procedure yielded similar results if the buffer volumes were adjusted when starting with either 200 mg or 1 g (dry weight) of bacteria. In addition, we ob-

tained LPS from 20 g of cells by making our first solubilization step in 200 ml of 2% SDS–0.1 M tetrasodium EDTA dissolved in 10 mM Tris-hydrochloride (pH 8) and adjusting other volumes accordingly. Our yield and purity were the same as previously described. We have also obtained LPS from 5 ml of midlogarithmic culture cells of *Yersinia enterocolitica* by adjusting our solubilization buffer to 200 μl (R. P. Darveau et al., manuscript in preparation).

(ii) Heating step before the second pronase treatment. With *P. aeruginosa*, the LPS preparation had less than 5% protein before heat treatment; with *E. coli*, however the preparation contained over 30% protein consisting mainly of porin protein with some Braun's lipoprotein being present. We found that when the properties of the SDS-resistant (pronase-resistant) proteins were not known, 30 min at 85°C was sufficient to ensure complete protein removal by subsequent steps. With the *P. aeruginosa* strains used in this study, it was necessary to heat in the presence of alkaline pH. Since this treatment may have caused a partial saponification of the ester-linked fatty acids, we examined the molar ratios of the fatty acids in the final LPS product with and without heat treatment in the presence of alkaline conditions. The results indicated that less than 30% of the 3-OH $C_{10:0}$ (i.e., 6% of the total LPS-linked fatty acid) was lost during this treatment but that no other LPS fatty acids were saponified. This is less than the total release of ester-linked fatty acids observed by two groups of researchers for the hot phenol-water procedure applied to *Serratia marcescens* (48) and *E. coli* (30). With *S. typhimurium* and *E. coli*, heating at neutral pH followed by pronase action (at elevated pH) was sufficient for protein removal.

(iii) Miscellaneous comments. The high EDTA concentrations in the procedure were essential for protein removal. When 5 to 50 mM EDTA was substituted, the LPS yields were equally high, but protein contamination of the final product increased. We believe the high concentrations of EDTA were required to completely dissociate the LPS molecules, allowing pronase to act on proteins which otherwise may be masked by LPS.

We tested by gas-liquid chromatography the pellets and dried supernatants for the loss of *P. aeruginosa* LPS at each stage of the purification. We also looked for the presence of LPS by quantitative rocket immunoelectrophoresis by using a monoclonal antibody (MA1–8) specific for LPS. Less than 1% of the cellular LPS was found in any of the discarded fractions, and we consider that our major losses were nonspecific and due to sticking to vessels, pipettes, etc.

Although the precipitation of LPS by ethanol (22, 49) and the aggregation of LPS in the presence of Mg^{2+} (31) is well documented, we found it necessary to combine these observations in our method to ensure good yields. A diagnosis for rough-type LPS in our isolation procedure was that within the first 10 min after the addition of the $MgCl_2$-ethanol solution, a white granular precipitate appeared. With smooth-type LPS, the appearance of a white precipitate usually took longer and it was not granular.

DISCUSSION

For obtaining LPS from rough-type organisms, the procedure of Galanos et al. is considerably more effective than the procedure of Westphal and Jann. However, smooth-type LPS is at least partially excluded in the Galanos procedure (8, 9, 19). In Table 2, the hydroxy fatty acid data from Table 1 was used to calculate the yield of LPS as a percentage of the cell (dry weight). We then compared our procedure with the procedures of both Galanos et al. and Westphal and Jann to demonstrate that not only does our procedure result in higher yields for most organisms examined, but it is universally applicable to both smooth and rough strains. In addition, the amount of LPS obtained from *P. aeruginosa* strains was substantially increased.

The procedure allowed us to make direct comparisons of LPS preparations obtained from wild-type and mutant strains. Examples presented here included the comparison of LPS obtained from strain K 799 and its antibiotic supersusceptible mutant Z61. Previous studies with these organisms (1, 20) strongly indicated that there was an LPS alteration in the mutant strain; however, owing to the low yield of LPS obtained from mutant Z61, we considered here the possibility that the previously used hot phenol-water extraction procedure was subfractioning the LPS. In this study, we clearly demonstrated that we greatly overestimated, in our previous study (20), the number of O-antigen side chains attached to the rough core of mutant Z61. In fact, the mutant Z61, despite its sensitivity to smooth LPS-specific phages, resistance to a rough-specific phage, lack of autoagglutination, and smooth colony appearance (20), has LPS that looks substantially like the LPS from the rough mutant AK 1121 which has the opposite phenotype. Only when we overloaded gels could we confirm the previous observation (20) of a heterogeneous smooth LPS population in mutant Z61 LPS. This provides a clear demonstration of the disadvantages of using an isolation technique which favors one type of LPS over another, since it is known that the hot phenol-water method is more effective for smooth than for rough organisms (Table 2).

In another example, a rough mutant of *S. typhimurium* LT2, designated *rfaH* 481, was found to contain completed LPS molecules with attached O-antigenic side chains of various lengths. This is of some interest because previous studies with this mutant have indicated the mutation results in an LPS that is heterogeneous (23). However, the authors stated they were unable to conclude whether completed LPS molecules were present, partly because of the method of LPS isolation (23).

It is known that a variety of gram-negative organisms, including *P. aeruginosa*, have anionic capsular polysaccharides (45). In addition, other polysaccharide components including enterobacterial common antigen and peptidoglycan fragments are potential contaminants of LPS in our procedure. However, since each of these products is water soluble (24, 25, 45), with the possible exception of some types of enterobacterial common antigen (25), these would remain in the supernatant upon ultracentrifugation as documented by Luderitz et al. (24). Furthermore, enterobacterial common antigen is ethanol soluble (25) and would thus not be expected to contaminate LPS after two cycles of ethanol precipitation.

A variety of immunochemical analyses performed on LPS obtained by our procedure suggested that the antigenic structure of the LPS was intact (11). Furthermore, by using certain preparations of *P. aeruginosa* LPS isolated here, J. Lam (Ph.D. thesis, University of Calgary, Canada, 1983) demonstrated that our LPS gave a single precipitin line in crossed immunoelectrophoresis studies by using antiwhole *P. aeruginosa* sera as the antibody source, suggesting that our LPS was not contaminated with other *P. aeruginosa* antigens.

A potential problem associated with our isolation procedure is the heating step at alkaline pH which was required for complete protein removal from *P. aeruginosa* but not for the other strains used in this study. This step could cause partial transesterification of ester-linked fatty acids, although generally speaking more rigorous conditions (e.g., 30 min, 100°C, pH 13) are required for substantial saponification. We noted a partial loss of one of the four species of fatty acids associated with *P. aeruginosa* LPS preparations (see above). However, we feel that the advantages of obtaining a more representative sample of the cellular LPS outweighs this disadvantage. Indeed, the hot phenol-water technique may also give rise to some degradation of LPS during the isolation procedure, including the loss of ester-linked fatty acids (30, 48).

With the continuing interest in the heterogeneity of LPS in gram-negative bacteria and the rising interest in examining the LPS composition of nonenteric organisms, our procedure offers the advantage that it provides the possibility of obtaining a more representative sample of the LPS. Furthermore, its applicability to small volumes of cells may well prove of value in genetic experiments involving LPS regulation.

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LITERATURE CITED

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