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Characterization of Lipopolysaccharides from *Escherichia coli* K-12 Mutants

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Chemical analyses of the carbohydrate composition of lipopolysaccharides (LPS) from a number of LPS mutants were used to propose a schematic composition for the LPS from *Escherichia coli* K-12. The formula contains four regions: the first consists of lipid A, ketodeoxyoctonoic acid, and a phosphorous component; the second contains only heptose; the third only glucose; and the fourth additional glucose, galactose, and rhamnose. LPS from *E. coli* B may have a similar composition but lacks the galactose and rhamnose units. A set of LPS-specific bacteriophages were used for comparing three mutants of *Salmonella* with a number of LPS mutants of *E. coli* K-12. The results confirm that there are basic similarities in the first and second regions of the LPS structure; they also support the four region divisions of the LPS formula. Paper chromatography was used for characterization of ^{32}P-labeled LPS from different strains of *E. coli* and *Salmonella*. The R_f values for LPS varied from 0.27 to 0.75 depending on the amounts of carbohydrates in the molecule. LPS from all strains studied was homogenous except for strain D31 which produced two types of LPS. Mild acid hydrolysis of labeled LPS liberated lipid A and two other components with phosphate, one of which was assigned to the first region. It is suggested that paper chromatography can be used in biosynthetic studies concerning regions 2 to 4.

The lipopolysaccharides (LPS) from gram-negative bacteria are known to be complex molecules consisting of three main parts: lipid A, the core polysaccharide, and the side chain polysaccharides; the latter is often referred to as O antigen (17). Most of the vast literature on LPS has been motivated by the endotoxic and immunogenic properties of the molecule (17). This is especially true for *Salmonella*, where the isolation of mutants missing parts of the O antigen or the core polysaccharide has facilitated the structural analysis of the LPS molecule (24, 36). Recently, the use of antibiotics and phage resistances provided means to obtain similar sets of mutants in *Escherichia coli* (8, 21, 38, 39).

Wild-type and class I ampicillin-resistant strains of *E. coli* K-12 (which lack O antigens) are normally resistant to the Wollman phage (ϕW), whereas a few class II and all class III ampicillin-resistant mutants are sensitive (3, 5, 8, 21). Starting from the latter class, resistance to ϕW was used to select mutants which had lost increasing amounts of the carbohydrates from the core of the LPS molecule (21). We have now isolated a new set of such mutants and made comparative analyses of the carbohydrate and the fatty acid composition of the LPS. It was found possible to use paper chromatography for the characterization of ^{32}P-labeled LPS from different mutants. Phosphorus containing degradation products obtained after mild acid hydrolyses were characterized in the same way. We have also compared the phage pattern for a number of *E. coli* and *Salmonella* mutants believed to have similar LPS structures. The results obtained by the different methods have been used for suggesting a formula for *E. coli* LPS. In a companion paper (4), we studied the phage-blocking capacity of LPS obtained from several of the strains here characterized.

MATERIALS AND METHODS

Bacterial strains and growth media. The origins and properties of the strains used are summarized in Table 1. Most strains of *E. coli* were from this laboratory and, for most of them, details of their properties are published (3, 5, 8, 21). Two new strains, D21f1 and D21f2, were isolated as follows. About 10^9 viable cells from an over-night culture of strain D21e7 were spread on an LA plate (see below) with about 4×10^9 plaque-forming units of phage ϕW. After an over night incubation at 37 C, the plate contained about 40 colonies with a variable morphology. Strain D21f1 was selected as rough, D21f2 as smooth, and both were

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TABLE 1. *E. coli and Salmonella strains used*[a]

Organism	Strain	Parental strain	Growth requirements	Known mutation(s) affecting LPS	Reference
E. coli B			None		
E. coli K-12	D21	D2	Pro, Trp, His		3, 21
E. coli K-12	D31	D3	Pro, Trp, His	Unknown 1	3, 21
E. coli K-12	D21e7	D21	Pro, Trp, His	*lpsA1*	8, 21
E. coli K-12	D215	D21	Pro, Trp, His	*lpsA2*	This paper
E. coli K-12	D31m3	D31	Pro, Trp, His	Unknown 1 + *galU*	21
E. coli K-12	D21f1	D21e7	Pro, Trp, His	*lpsA1* + unknown 2	This paper
E. coli K-12	D31m4	D31	Pro, Trp, His	Unknown 1 + unknown 3	21
E. coli K-12	D21f2	D21e7	Pro, Trp, His	*lpsA1* + unknown 4	This paper
S. typhimurium	SL869		Met, Trp	*galE*	12
S. typhimurium	SL1102		Met, Trp	*rfaE*	41
S. minnesota	R595		None	Heptose-less	15
E. coli K-12	GR467	CR34	Leu, Thr, Thy	Leaky heptose-less	29

[a] The parental strain D3 was obtained from D2. All strains of *E. coli* K-12 from this laboratory contain *ampA* and *strA*. The unknown *lps* genes seem to map near 70 min. Abbreviations: His, histidine; Met, methionine; Pro, proline; Thy, thymine; Thr, threonine; and Trp, tryptophan. Gene abbreviations are described in the respective references.

purified twice on LA plates and simultaneously tested for sensitivity to ϕ5. Strain D215 was isolated after mutagenesis with nitrosoguanidine and screening for sensitivity to phage ϕW. *E. coli* strain GR467 (29) was obtained from H. Goldfine. *Salmonella typhimurium*, strains SL 869 (12) and SL 1102 (41), originate from B. Stocker. The former was obtained from G. Schmidt, who also provided us with strain R595 of *Salmonella minnesota*.

Broth medium was made up of basal medium E (40) supplemented with Luria broth (1), 0.2% glucose, and thiamine (1 μg/ml). For strain GR467, it was also necessary to add thymine (10 μg/ml). Medium for 15-liter cultures were supplemented with 1.3% nutrient broth (Oxoid) and 0.1% tryptone (Difco). For preparation of ^{32}P-labeled LPS, the bacteria were pregrown in a low phosphate medium (11) supplemented with 0.2% Casamino Acids, tryptophane (20 μg/ml), 0.2% glucose, and 0.05 M morpholinopropane sulfonic acid (MOPS buffer) (Sigma Chemical Co.; Neidhardt, personal communication). Agar plates (LA) contained Luria broth supplemented with 1.5% agar, 2.5 mM $CaCl_2$, and a vitamin mixture (8). Soft agar for overlaying was Luria broth with 0.75% agar. DM medium for dilution of phages consisted of 0.1% nutrient broth (Oxoid) with 0.5% NaCl.

Bacteriophage strains. The Wollman phage, ϕW, and its host-range mutant, ϕ3, have been described (19, 42). Detailed characterization showed that ϕ3 is denser than the parental phage ϕW and that both ϕW and ϕ3 produced tailless particles when grown on strain D31 (20). Phage ϕ5 is a new host-range mutant of ϕW, isolated after plating twice on the heptose-less strain D31m4 and picking single plaques each time. It produces regular plaques, 1 to 2 mm in diameter, with a halo that develops after about 4 h of incubation at 37 C. Strain D31m4 was always used both for propagation and as indicator for phage ϕ5. Other phages were grown on *E. coli* B. Phage stocks were made from host cells infected during exponential growth in broth medium at 37 C. After chloroform treatment and removal of cell debris they were stored at 4 C. The *Salmonella* phage FP3 (34) was obtained from G. Schmidt and was maintained by propagation on strain R595 (Table 1). Phage C21 (6), kept in our collection for several years, was obtained from A. Lindberg. It was propagated on *E. coli* strain B. Phage C21-3 is a mutant obtained from C21 after selection on strain D31m4. It was propagated on strain D31m4 which was also used as standard indicator.

Preparation of LPS. Starting from lyophilized cells, LPS was prepared by the method of Galanos et al. (10) with the following minor modifications. To obtain a homogenous mixture of chloroform, petroleum ether, and water containing phenol (PCP), we normally reduced the water content to 7 ml per 90 g of phenol. For heptose-less LPS, the water precipitation was incomplete. With such LPS this step was therefore sometimes substituted by precipitation with 2 volumes of acetone (15). The last step in the procedure was ultracentrifugation (10). These LPS preparations were found to have the same phage-blocking capacity (4) and carbohydrate composition as material prepared strictly according to Galanos et al. (10). However, the fatty acid contents were different and they often contained more palmitic acid. LPS solutions were always stored frozen and, to solubilize the material after thawing, they were heated to about 55 C for 5 min.

Preparation of ^{32}P-labeled LPS. To 10 ml of low phosphate medium was added about 0.2 mCi of ^{32}P as sterile orthophosphate in HCl solution (PBS-1 from the Radiochemical Centre LTD, Amersham, England) and carrier phosphate to a final concentration of 2×10^{-4} M. This medium was inoculated with about 5×10^7 cells in 0.1 ml, pregrown in low phosphate medium. The culture was incubated at 37 C until reaching 3 to 4 $\times$ 10^8 cells/ml. At this cell density the phosphate of the medium was nearly depleted. The cells were harvested by centrifugation, washed with 5

ml of water and 5 ml of acetone, and finally dried by suction of air over the surface of the pellet. Carrier cells were prepared from a 20-ml culture in luria broth medium treated in the same way. The carrier cells were mixed with 1 ml of PCP and the suspension was transferred to the ^{32}P-labeled cells and repeatedly shaken for 10 min. The bacterial residues were removed by centrifuging at 3000 × *g* for 15 min at 22 C. The supernatant was collected (the precipitate discarded) and most of the chloroform and petroleum ether was evaporated by a gentle suction of air over the surface of the PCP mixture. To the remaining volume (0.2 to 0.3 ml) was added 1.5 ml of ice-cold acetone. The mixture was left in ice for at least 10 min and then centrifuged at 4000 × *g* for 15 min at 4 C. The supernatant was discarded and the precipitate was dissolved in 0.5 ml of water and heated to 50 C for 3 min. After cooling in ice, it was reprecipitated twice with1.5 ml of ice-cold acetone. The samples so obtained contained 2 × 10 to 5 × 10^6 counts/min in 0.5 ml. They were usually diluted five to ten times when used for acid hydrolyses or chromatography.

Analytical methods. Carbohydrate analyses of LPS were performed by the gas-chromatographic method of Hellerqvist et al. (14) with the modifications previously used (21).

Hydrolysis for determination of fatty acids was carried out in sealed ampoules containing 5 to 10 mg of LPS in 3 to 4 ml of methanol with a 6% concentration of HCl (15). After cooling, the mixture was neutralized with NaOH and the methyl esters of the fatty acids were taken up in redistilled petroleum ether, using three successive extractions. The pooled petroleum ether fractions were then evaporated to a small volume and a sample was analyzed with a Perkin-Elmer model 900 gas chromatograph. Since no internal standards were used, only the relative content of the different fatty acids could be determined and not the absolute amounts.

Mild acid hydrolysis of LPS was a minor modification of the standard procedure (17). To 25 μliters of ^{32}P-labeled LPS was added an equal volume of 0.3 M acetic acid adjusted to pH 3.5 with triethylamine. The samples were heated to 100 C for 20 min, then cooled in ice. The lipid A liberated was removed by centrifugation at 3000 × *g* for 15 min at 4 C. The supernatant was taken up in a capillary tube and lipid A was dissolved in chloroform-methanol (4:1).

Paper chromatography of ^{32}P-labeled LPS and hydrolysis products was performed in the following ways: strips of Whatman paper 3MM (10 or 20 cm by 23 cm) were developed by ascending isobutyric acid-1 M ammonium hydroxide, either 7:3 (system A) or 5:3 (system B). The latter system is widely used for the separations of murein intermediates (25) and was "accidentally" found suitable also for LPS. For radioautograms the amount of radioactivity normally applied to each spot was about 5,000 counts/min in 5 to 10 μliters. The LPS solution applied could be dried by a stream of cold air but heating must be avoided. After development, the chromatograms were dried and heated to 85 C for 10 min, then exposed overnight to a highly sensitive industrial X-ray film (Test-X H, Ceaverken, Strängnäs, Sweden). It was also possible to chromatograph the undiluted LPS samples and expose on X-ray film for 1.5 to 2 h.

RESULTS

Composition of E. coli K-12 LPS based on carbohydrate analyses of LPS from key mutants. The formula to be presented should be considered as a working hypothesis. The backbone consists of data from four bacterial strains, the parent D21 and the three mutants D21e7, D21f1, and D21f2, derived as indicated by arrows with filled heads in Fig. 1. Carbohydrate analyses of LPS from these strains (Table 2) show that they have lost increasing amounts of sugar residues as indicated by the four regions separated by vertical broken lines and arrows with open heads in Fig. 1. Strain D21e7 carries the *lpsA* mutation at 72 min characterized before (8). Later we isolated a second mutant, D215, which by all available criteria also carries a mutation in the *lpsA* gene. Earlier, from strain D31 (here shown superficially to behave as a leaky *lpsA* mutant), we isolated and characterized two spontaneous mutants, D31m3 and D31m4, selected as resistant to the Wollman phage (21). The two new mutants D21f1 and D21f2 were found to have an LPS of a

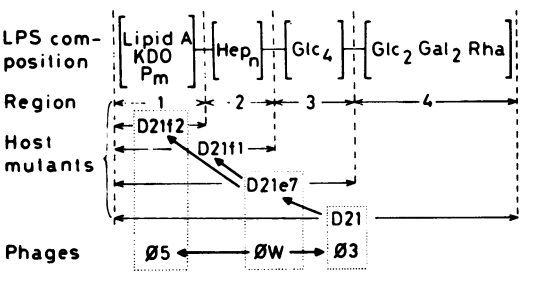

FIG. 1. *Suggested formula for the composition of LPS from E. coli K-12 deduced from chemical analysis of LPS from four interrelated mutants (data given in Table 2). The four regions are believed to reflect defined steps in the biosynthesis of the LPS. The numbers for the individual residues are based on the assumption that each chain contains 1 unit of rhamnose. Strain D21e7 carries a mutation in the lpsA gene. It was obtained from strain D21 as a spontaneous class II ampicillin-resistant mutant. Strains D21f1 and D21f2 were isolated from strain D21e7 as spontaneous mutants resistant to the Wollman phage ϕW and sensitive to the host-range mutant ϕ5. These phages and another host-range mutant, ϕ3, are inserted in the bottom part of the figure. Each phage and its corresponding host are surrounded by a square of dotted lines. Arrows with filled heads indicate mutational steps. Chemical abbreviations: KDO, ketodeoxyoctonoic acid; Hep, heptose; Glc, glucose; Gal, galactose; Rha, rhamnose; P, phosphate.*

TABLE 2. *Carbohydrate analysis of LPS from E. coli K-12 mutants*[a]

Source of LPS		Carbohydrates (μg/mg of LPS)					
Strain	Growth	Rib	Hep	Glc	Gal	Rha	GlcN
E. coli B	Log	2.1	126	90	<0.1	<0.1	33
E. coli B	Stat	1.3	107	83	<0.1	<0.2	28
D21	Log	1.1	117	94	39	16	31
D21	Stat	0.7	108	81	35	15	20
D31	Log	3.5	118	73	12	5	32
D31	Stat	0.5	79	64	19	2	15
D215	Log	1.2	112	62	2.2	<0.1	27
D215	Stat	0.9	111	70	2.5	<0.2	28
D21e7	Log	<2	91	49	<2	<0.5	
D31m3	Log	<1	115	10	<1	<0.2	
D21f1	Log	0.5	105	1	<0.5	<0.5	
D31m4	Log	0.9	1.1	<0.5	<0.1	<0.1	30
D21f2	Log	0.2	0.1	<0.1	<0.1	<0.2	

[a] Gas chromatographic analysis according to Hellerqvist et al. (14) using xylose as internal standard. Glucosamine, the last compound to be eluted, was partly decomposed during the analysis. The data are included only as an indicator of a suspected variable destruction also of glucose and heptose. Abbreviations: Rib, ribose; Hep, heptose; Glc, glucose; Gal, galactose; Rha, rhamnose; GlcN, glucosamine.

composition similar to D31m3 and D31m4, respectively (Table 2).

Phages of different origin have frequently been used for the grouping of different LPS mutants (26). We have used here a parental phage, ϕW, and two host-range mutants, ϕ3 and ϕ5. These three phages and their respective hosts are in Fig. 1 enclosed by rectangles of dotted lines. The specificity of the phages will be discussed below as well as in the companion paper (4).

Paper chromatography of intact and degraded ^{32}P-labeled LPS. It was found possible to characterize ^{32}P-labeled LPS by use of paper chromatography. Figure 2 shows a chromatogram developed in system A with LPS from eight different strains of *E. coli* and *Salmonella*. The lowest R_f values, 0.27 and 0.37, were found for LPS from strains D21 and *E. coli* B, which according to our carbohydrate analysis in Table 2 have the largest polysaccharide moieties. The highest R_f value, 0.75, was found for LPS from three heptose-less mutants, strains D21f2, D31m4 of *E. coli* K-12, and strain R595 of *S. minnesota*. However, in this case, the R_f was not only a product of the solubility of the LPS but also the result of a "second front" which travelled through the paper (2). Other LPS molecules with intermediary R_f values were unaffected by this "second front". Strain D31 gave two spots with R_f values 0.22 and 0.53 which correspond to the parental strain D21 (0.27) and to the *lpsA* mutant D21e7 (0.56). Also, for the latter strain, very small amounts of wild-type LPS could be detected.

^{32}P-labeled LPS from some of the mutants was characterized by a study of the products obtained after a mild acid hydrolysis at pH 3.5 and 100 C for 20 min (see above). As illustrated in Fig. 3, this degradation produced a water-soluble polysaccharide fraction and a lipid A-containing precipitate which could be removed by centrifugation. The water-soluble fraction from seven different strains was compared by paper chromatography as shown in Fig. 4. Three of the key strains in Fig. 1, D21e7, D21f1, and D21f2, together with the *Salmonella* mutant R595, gave a single, well-defined band with the same R_f value of about 0.36. Quantitative determinations of the radioactivity showed that the water-soluble material from the three strains of *E. coli* K-12 accounted for 12 to 19% of the total phosphorus of the LPS, whereas the corresponding figure for *S. minnesota*, strain

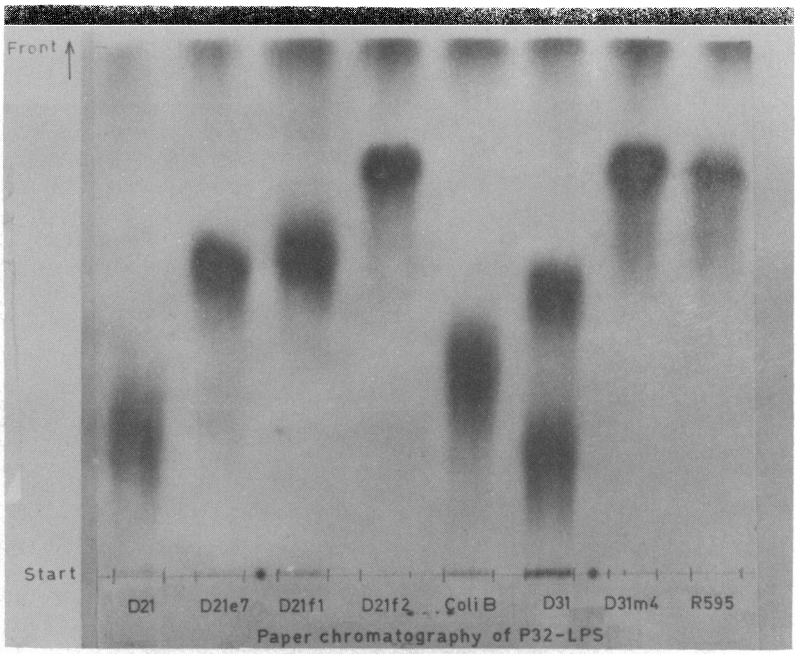

FIG. 2. *A paper chromatogram with ^{32}P-labeled LPS prepared from eight different strains. From left are first LPS from the four key strains of Fig. 1: D21, D21e7, D21f1, and D21f2. Number 5 is LPS prepared from E. coli B. Number 6 is LPS from stationary-phase cells of strain D31. Log-phase cells of strain D31 contained considerably larger proportion of the faster component. Number 7 and 8 are LPS from two heptose-less mutants, D31m4 from E. coli, and R595 from S. minnesota. More material than normal was applied to this chromatogram to demonstrate the homogeneity. The impurities moving with the front were most likely phosphatidylethanolamine and phosphatidylglycerol. Whatman 3MM was used with solvent system A (isobutyric acid-1 M ammonium hydroxide [7:3]). The radioautogram was made by a 15-h exposure to a highly sensitive X-ray film.*

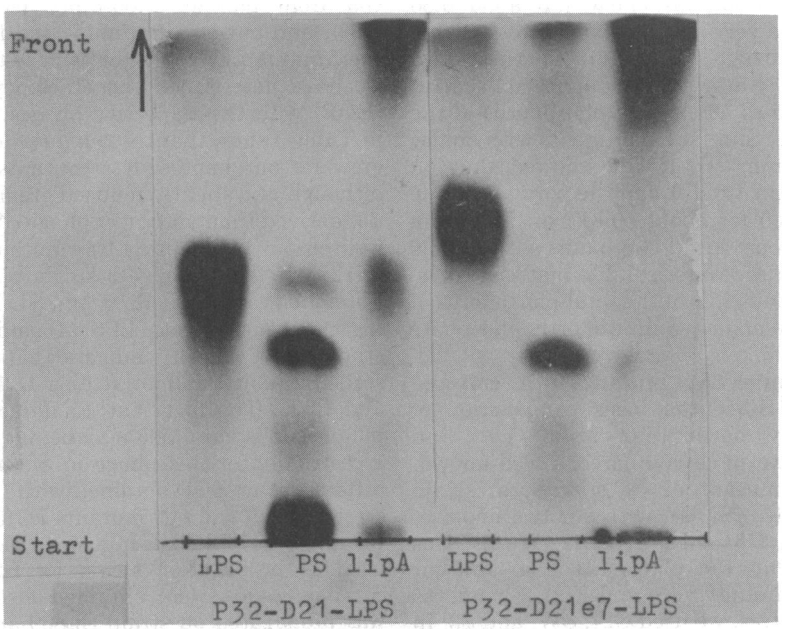

FIG. 3. *Two paper chromatograms illustrating the mild acid hydrolysis used (as in Material and Methods except that 0.1 M acetic acid was used). Left part: ^{32}P-labeled LPS from strain D21 before the hydrolysis (LPS); the water-soluble fraction obtained after hydrolysis and centrifugation (PS); and the precipitated lipid A (lipA) redissolved in chloroform methanol (4:1). Right part: the corresponding fractions for ^{32}P-labeled LPS from strain D21e7. The hydrolysis of D21 LPS was not quite complete with 0.1 M acetic acid and traces of intact LPS were present both in the water-soluble and precipitated fraction. The paper was Whatman 3MM and the solvent system isobutyric acid-1 M ammonium hydroxide (5:3), system B.*

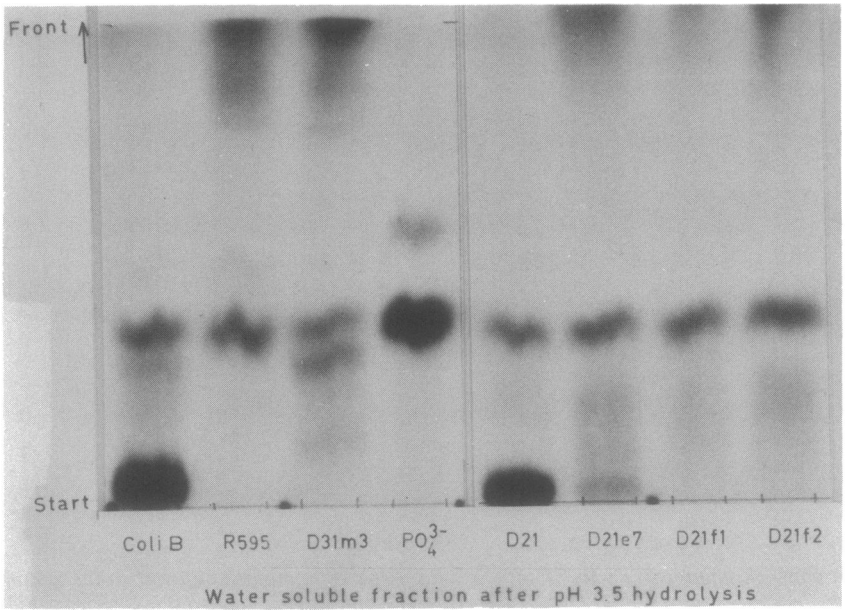

FIG. 4. *Two chromatograms of the water-soluble fraction obtained after mild acid hydrolysis (0.15 M acetic acid as in Material and Methods) of LPS from seven different strains. From left are spots with material from E. coli B, S. minnesota, R595, and E. coli K-12 strain D31m3. Spot number 4 is a reference sample of ^{32}P-labeled orthophosphate. The right chromatogram contains material from the four key strains of Fig 1: D21, D21e7, D21f1, and D21f2. The paper was Whatman 3MM and solvent system B was used.*

R595, was about 40%. LPS from strains D21 and *E. coli* B gave two bands, one with R_f about 0.36 and one with a very low but significant R_f value (0.03 and 0.04, respectively). In these two strains, the water-soluble material corresponded to 55 to 58% of the phosphorus of the LPS molecule. Similar experiments with nonlabeled LPS from strain D21 showed that on weight basis the lipid A and the polysaccharide part accounted for about 50% each. LPS from strain D31m3 produced two bands with R_f 0.36 and 0.30, and the water-soluble material corresponded to about 25% of the total phosphorus of this LPS. A reference sample of orthophosphate showed R_f 0.37.

Comparison of LPS mutants of E. coli and Salmonella. Since the basis composition of lipid A may be similar in *E. coli* and *Salmonella*, we have compared a well-known, heptose-less mutant of *S. minnesota*, strain R595 (7, 13, 15, 28, 34), with our two heptose-less mutants, D31m4 and D21f2. We used as tools the *Salmonella* phage FB3 (34) and our own phage mutants ϕ5 and C21-3, and, as references phages, we used C21, ϕW, and ϕ3. In the comparison, we included two strains of *S. typhimurium*, a heptose-less *rfaE* mutant (strain SL 1102 [41]) and a uridine 5'-diphosphate-galactose-epimerase-less mutant (strain SL 869 [12]), a leaky heptose-less mutant of *E. coli* from another laboratory (strain GR467 [29]), and our own mutants used for deducing the formula in Fig. 1. For each of these strains we have determined the efficiency of plating (EOP) with the respective phages. The results in Table 3 show that the *Salmonella* phage FP3 grew on our heptose-less mutants of *E. coli*, although at a slightly reduced efficiency. Phage ϕ5, derived from an *E. coli* phage ϕW (42), grew well on our two heptose-less mutants of *E. coli* K-12, but did not give a single plaque on the *Salmonella* mutants R595 and SL 1102. Blocking experiments with LPS (4) and adsorption studies (not shown) indicate that ϕ5 was adsorbed equally well to strains D21f2, D31m4, and R595. The fact that ϕ5 did not give any plaques on strain R595 may be due therefore to a restriction of this phage in *Salmonella*. The differences in EOP obtained with C21 between strain SL869 and our mutants D215 and D21e7 (Table 3) were unexpected since SL869 is often used as propagation strain for C21 (32, 33). Similar results were obtained also when C21 was propagated on strain SL869 and a restriction effect is therefore unlikely.

Compared to phages C21 and ϕW (isolated 40 and 27 years ago, respectively), our host-range mutants ϕ3, ϕ5, and C21-3 are young and they

TABLE 3. *Comparative EOP values for Salmonella and E. coli LPS mutants*[a]

Host		Phages					
Strain	LPS	FP3	φ5	C21-3	C21	φW	φ3
SL869	1-3	10^{-4}	$<10^{-9}$	10^{-8}	0.05	10^{-8}	$<10^{-10}$
SL1102	1	1	$<10^{-8}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$
R595	1	1	$<10^{-9}$	$<10^{-9}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$
D31m4	1	0.03	1	0.5	0.02	10^{-9}	0.5
D21f2	1	0.1	1	0.5	0.04	10^{-8}	0.5
D31m3	1-2	10^{-4}	1	1	0.2	10^{-8}	1
D21f1	1-2	10^{-4}	1	1	0.3	10^{-8}	1
D215	1-3	10^{-5}	0.1	1	1	1	1
D21e7	1-3	10^{-5}	0.2	1	1	1	1
D31	1-3(4)	10^{-5}	0.1	1	1	1	1
D21	1-4	10^{-4}	10^{-5}	1	0.03	10^{-9}	0.5
GR467	1-(2-4)	10^{-5}	0.4	1	0.01	10^{-8}	0.6

[a] The top three strains are mutants of *Salmonella*, others are derivatives of *E. coli* K-12. The LPS numbers refer to the regions of the proposed LPS formula in Fig. 1. Parenthesis indicates synthesis also of enclosed regions. Propagation strains for the phage are given in Materials and Methods.

may be somewhat unstable in their host-range properties (6, 19, 20, 42). The data in Table 3 were reproducible within a few months, but they do not fully agree with experiments performed a year or more back. Another source of error comes from the fact that many poor host strains tend to give a variable plaque size with an accompanying risk for overlooking small plaques. The EOP values given here are believed therefore to be comparable with each other but not suitable as lasting reference data.

Fatty acid analysis of LPS from different strains. The fatty acid composition is one means to characterize the lipid A part of the LPS molecule. We have analyzed therefore some of our LPS preparations and compared the results with data available for the *Salmonella* mutant R595 (15, 28) (Table 4). Allowing for certain errors, the ratio between the three main components C_{12}, C_{14}, and β-hydroxymuristic acid would be 1:1:2, whereas palmitic acid (C_{16}) must be considered only a minor component in our strains of *E. coli* K-12. In contrast are the two analyses of LPS from the mutant R595 of *S. minnesota*, where ratios 1:1:1:3 were given (28) with palmitic acid as one of six residues. For comparison we included also published data for heptose-less LPS from GR467 (30), a leaky heptose-less mutant of *E. coli* K-12 (29).

TABLE 4. *Fatty acid composition of LPS from mutants of E. coli and Salmonella*[a]

Source of LPS		Fatty acids (% of total)			
Strain	Growth phase	C_{12}	C_{14}	C_{16}	βHM
E. coli K-12, D21	Log	32	25	2	40
E. coli K-12, D21	Stat	24	28	2	46
E. coli K-12, D31	Log	25	25	4	46
E. coli K-12, D31	Stat	25	26	2	47
E. coli B	Log	23	20	4	52
E. coli B	Stat	27	25	2	46
E. coli K-12, D215	Log	26	25	1	47
E. coli K-12, D31m4	Log	27	23	3	46
Salmonella, R595	ref. 15	27	25	<u>19</u>	30
Salmonella, R595	ref. 28	23	16	<u>16</u>	46
E. coli K-12, GR467	ref. 30	30	24	<u>13</u>	32

[a] All our LPS samples were prepared by water precipitation (10) even when this was incomplete (see Materials and Methods). Gas chromatography (14) was used for determinations of the following fatty acids: lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}), and β-hydroxymyristic acid (βHM). The sum of other components (not given) in LPS from our *E. coli* strains was always less than 1%. Increases believed to be significant are underlined. Upper part, strains of *E. coli* used here; lower part, published analyses for *Salmonella* and *E. coli* strains, recalculated to comparable data.

DISCUSSION

Homogeneity, purity, and analysis of LPS preparations. Shands in a recent review (35) states that "the lack of precise definition of LPS and the lack of good criteria for purity have hampered the study of its structure." His judgement was that the best purity criterion available was the ribose content, which we therefore included in Table 2. However, for heptose-less LPS, thin layer and ion exchange paper chromatography have been used as a purity criterion (15, 29). Our results in Fig. 2 show that paper

chromatography with system A can be used as a homogeneity and purity criterion also for LPS types with more carbohydrates. Preliminary experiments indicate that system B may give an improved resolution for some types of LPS with low R_f values in system A. With one exception (strain D31, to be discussed elsewhere), our LPS preparations studied here were found to be more than 80% homogeneous with respect to phosphorus. ^{32}P-labeled LPS was always free of orthophosphate but contained some phospholipid impurities, probably phosphatidylethanolamine and phosphatidylglycerol (15, 29). No additional impurities could be detected in ^{14}C-labeled LPS from strains D21e7 and D21f2 grown on uniformly ^{14}C-labeled glucose.

The PCP extraction of Galanos et al. (10) is milder and gives much less impurities than the hot-phenol-water method used before. This difference in LPS preparation may be the main reason why the glucose and heptose values in Table 2 are higher than those reported earlier (21). However, it is known that heptose decomposes during the hydrolysis and the gas chromatography (21, 32), and we believe our present heptose values still are too low. Since in *E. coli* K-12 glucosamine is only present in the lipid A part of LPS (26), we should expect, for strains with decreasing amounts of carbohydrates, increasing proportions of this component. The variations seen in Table 2 may therefore reflect a variable destruction of both glucosamine as well as other components during the hydrolysis at 100 C for 18 h with 0.25 M H_2SO_4. Rapin has made similar observations (personal communication).

How equal are LPS from Salmonella and E. coli K-12 and B? Structural studies of heptose-less LPS from *E. coli* (29, 30) and from *Salmonella* (13, 15, 28) indicate that there must be basic similarities both in the lipid A and in the ketodeoxyoctonoic acid parts of the molecule (7, 22). The innermost part of the core (regions 2 and 3 in Fig. 1) may also show basic similarities as evident from chemical analysis of LPS (17, 22) and from the sensitivity to phage C21 (8, 26, 27, 31, 33). Although several species differences have been reported (9, 18, 22, 31, 32), our results in Table 3 cannot be safely assigned to LPS, because for ϕ5 there may be a restriction effect, and for phages C21-3 and FP3 we have failed to obtain conclusive blocking experiments.

Table 2 shows that there was a qualitative similarity in the carbohydrate composition between LPS from our two *lpsA* mutants, D21e7 and D215, and *E. coli* B. However, LPS from the latter strain contains about the same amount of glucose as strain D21. LPS from *E. coli* B may therefore contain, in addition to regions 1–3, the glucose units of region 4, a suggestion consistent with both the R_f values of intact LPS (Fig. 2) and the pattern found after mild acid hydrolysis (Fig. 4).

Limitations of the proposed LPS formula. For the seven strains investigated, mild acid hydrolysis of ^{32}P-labeled LPS produced a degradation product with an R_f value similar to orthophosphate (Fig. 4). This material was obtained from all strains studied, and therefore, it must belong structurally to region 1. Since the further phosphorylation of LPS may require that regions 2 to 3 and part of region 4 were added (16), the main phosphorus-containing component present in the core part of the LPS from strains D21 and *E. coli* B (with very low R_f values in Fig. 3) cannot yet be assigned structurally to any part of the molecule.

It should be stressed that the LPS formula given in Fig. 1 is meant as a means to group now available mutants according to chemotype and phage sensitivity. The formula reflects the LPS composition together with the order by which regions 2 to 4 are added to the backbone of lipid A, but it does not necessarily imply chemical bonds between neighboring regions or sugar residues. The regular pattern for the EOP values in Table 3 indicates that the division of the LPS formula into four different regions also can have a physiological significance. However, yet undiscovered components may have to be added to account for "the missing percentage" (23). The formula is schematic because we do not know whether the molecule contains multiple chains of regions 2 to 4 attached to a backbone of region 1, nor do we know much about the arrangements of the individual sugars. However, the recent isolation of a rhamnosyl-ketodeoxyoctonoic acid fragment from strain D21 (Rapin, personal communication) indicates that rhamnose in our strains is linked to ketodeoxyoctonoic acid as previously reported for another derivative of *E. coli* K-12 (37). Finally, it is evident that LPS from the different mutants, together with paper chromatography as shown in Fig. 2, will provide a simple method for an in vitro study of the biosynthetic reactions involved in the addition of regions 2 to 4 of the LPS molecule.

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LITERATURE CITED

1. Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62**:293-300.
2. Boman, H. G. 1952. "Double fronting" in paper chromatography of proteins. Nature (London) **170**:703.
3. Boman, H. G., S. Jonsson, D. Monner, S. Normark, and G. Bloom. 1971. Cell-surface alterations in *Escherichia coli* K-12 with chromosomal mutations changing ampicillin resistance. Ann. N.Y. Acad. Sci. **182**:342-357.
4. Boman, H. G., and D. A. Monner. 1974. Blocking of bacteriophages ϕW and ϕ5 with lipopolysaccharides from *Escherichia coli* K-12 mutants. J. Bacteriol. **121**:465-470.
5. Boman, H. G., K. Nordström, and S. Normark. 1974. Penicillin resistance in *Escherichia coli* K12: synergism between penicillinases and a barrier in the outer part of the envelope. Ann. N.Y. Acad. Sci. **235**:569-585.
6. Burnet, F. M. 1933. The classification of dysentery-coli bakteriophages. II. The serological classification of coli-dysentery phages. J. Pathol. Bacteriol. **36**:307-318.
7. Dröge, W., V. Lehmann, O. Lüderitz, and O. Westphal. 1970. Structural investigations on the 2-keto-3-deoxyoctonate region of lipopolysaccharides. Eur. J. Biochem. **14**:175-184.
8. Eriksson-Grennberg, K. G., K. Nordström, and P. Englund. 1971. Resistance of *Escherichia coli* to penicillins. IX. Genetics and physiology of class II ampicillin-resistant mutants that are galactose negative or sensitive to bacteriophage C21, or both. J. Bacteriol. **108**:1210-1223.
9. Fuller, N. A., M.-C. Wu, R. G. Wilkinson, and E. C. Heath. 1973. The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. VII. Characterization of heterogeneous "core" oligosaccharide structures. J. Biol. Chem. **218**:7938-7950.
10. Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9**:245-249.
11. Garen, A., and C. Levinthal. 1960. A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta **38**:470-483.
12. Gemski, P., Jr., and B. A. D. Stocker. 1967. Transduction by bacteriophage P22 in nonsmooth mutants of *Salmonella typhimurium*. J. Bacteriol. **93**:1588-1597.
13. Gmeiner, J., O. Lüderitz, and O. Westphal. 1969. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 6. Investigations on the structure of the lipid A component. Eur. J. Biochem. **7**:370-379.
14. Hellerqvist, C. G., B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg. 1968. Structural studies on the O-specific side chains of cell wall lipopolysaccharide from *Salmonella typhimurium* 395MS. Carbohydr. Res. **8**:43-55.
15. Kasai, N., and A. Nowotny. 1967. Endotoxic glycolipid from a heptose-less mutant of *Salmonella minnesota*. J. Bacteriol. **94**:1824-1836.
16. Lehmann, V., O. Lüderitz, and O. Westphal. 1971. The linkage of pyrophosphorylethanolamine to heptose in the core of *Salmonella minnesota* lipopolysaccharides. Eur. J. Biochem. **21**:339-347.
17. Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido. 1971. Characterization of bacterial lipopolysaccharides, p. 145-233. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
18. Medearis, D. N., Jr., B. M. Camitta, and E. C. Heath. 1968. Cell wall composition and virulence in *Escherichia coli*. J. Exp. Med. **128**:399-414.
19. Monner, D. A., and H. G. Boman. 1970. Female strains of *Escherichia coli* K12 selective hosts for the isolation of female specific mutants of phage ϕII. Biochem. Biophys. Res. Commun. **39**:1017-1020.
20. Mönner, D. A., and H. G. Boman. 1974. Host dependent properties of coliphage ϕW and its female specific host-range mutants, ϕ3 and ϕ4. J. Gen. Virol. **24**:67-75.
21. Monner, D. A., S. Jonsson, and H. G. Boman. 1971. Ampicillin-resistant mutants of *Escherichia coli* K-12 with lipopolysaccharide alterations affecting mating ability and susceptibility to sex-specific bacteriophages. J. Bacteriol. **107**:420-432.
22. Morton, J. J., and J. C. Stewart. 1972. Structural studies on the lipopolysaccharide of a rough strain of *Escherichia coli*. Eur. J. Biochem. **29**:308-318.
23. Nowotny, A. 1971. Chemical and biological heterogeneity of endotoxins, p. 309-329. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
24. Osborn, M. J., and L. I. Rothfield. 1971. Biosynthesis of the core region of lipopolysaccharide, p. 331-350. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
25. Park, J. T., and A. N. Chatterjee. 1966. Membrane associated reactions involved in bacterial cell wall mucopeptide synthesis, p. 466-472. *In* E. F. Neufeld and V. Ginsberg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
26. Rapin, A. M. C., and H. M. Kalckar. 1971. The relation of bacteriophage attachment to lipopolysaccharide structure, p. 267-307. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
27. Rapin, A. M. C., H. M. Kalckar, and L. Alberico. 1968. The metabolic basis for masking of receptor-sites on *E. coli* K-12 for C21, a lipopolysaccharide core-specific phage. Arch. Biochem. Biophys. **128**:95-105.
28. Rietschel, E. T., H. Gottert, O. Lüderitz, and O. Westphal. 1972. Nature and linkages of the fatty acids present in the lipid-A component of *Salmonella* lipopolysaccharides. Eur. J. Biochem. **28**:166-173.
29. Rooney, S. A., and H. Goldfine. 1972. Isolation and characterization of 2-keto-3-deoxyoctonate-lipid A from a heptose-deficient mutant of *Escherichia coli*. J. Bacteriol. **111**:531-541.
30. Rooney, S. A., H. Goldfine, and C. C. Sweeley. 1972. The identification of trans-2-tetradecenoic acid in hydrolysates of lipid A from *Escherichia coli*. Biochim. Biophys. Acta **270**:289-295.
31. Schmidt, G. 1973. Genetical studies on the lipopolysaccharide structure of *Escherichia coli* K12. J. Gen. Microbiol. **77**:151-160.
32. Schmidt, G., I. Fromme, and H. Mayer. 1970. Immunochemical studies on core lipopolysaccharides of enterobacteriaceae of different genera. Eur. J. Biochem. **14**:357-366.
33. Schmidt, G., B. Jann, and K. Jann. 1970. Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Studies on R mutants with an incomplete core, derived from *E. coli* 08:K27. Eur. J. Biochem. **16**:383-393.
34. Schmidt, G., and O. Lüderitz. 1969. Untersuchungen zur Typisierung von *Salmonella*-R-formen. 2. Mitteilung. Typisierung von *S. minnesota*-Mutanten durch Phagen. Zentralbl. Bakteriol. Parasitenk. Infectionskr. Hyg. Abt. Orig. **210**:381-387.
35. Shands, J. W., Jr. 1971. The physical structure of bacterial lipopolysaccharides, p. 127-144 *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
36. Stocker, B. A. D., and P. H. Mäkelä. 1971. Genetic aspects of biosynthesis and structure of Salmonella lipopolysaccharide, p. 369-438. *In* G. Weinbaum, S.

Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
37. Sugimoto, K., and R. Okazaki. 1967. A new rhamnosyl compound in lipopolysaccharide preparations of *Escherichia coli*. J. Biochem. (Tokyo) **62**:373-383.
38. Tamaki, S., and M. Matsuhashi. 1973. Increase in sensitivity to antibiotics and lysozyme on deletion of lipopolysaccharides in *Escherichia coli* strains. J. Bacteriol. **114**:453-454.
39. Tamaki, S., T. Sato, and M. Matsuhashi. 1971. Role of lipopolysaccharides in antibiotic resistance and bacteriophage adsorption of *Escherichia coli* K-12. J. Bacteriol. **105**:968-975.
40. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97-106.
41. Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker. 1972. Non-smooth mutants of *Salmonella typhimurium*: Differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70**:527-554.
42. Wollman, E. 1947. Relations entre le pouvoir de synthetiser la proline et la resistance au bacteriophage chez des mutants d'*Escherichia coli*. Ann. Inst. Pasteur Paris **73**:348-363.