ON THE PRIMARY STRUCTURE OF THE ESCHERICHIA COLI R4 CELL WALL LIPOPOLYSACCHARIDE CORE

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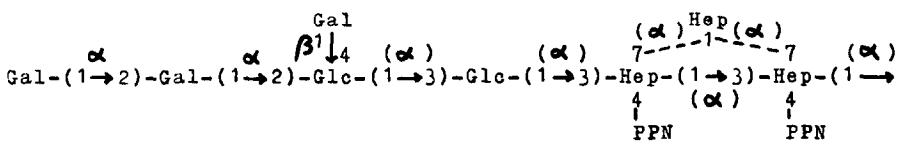
SUMMARY

From Escherichia coli R4, and from some deeper rough mutants of it, the cell wall lipopolysaccharides were isolated and subjected to mild acid hydrolysis. By methylation/g.l.c./m.s. and other analyses of the core oligosaccharides thus obtained, the primary structure of the E. coli R4 core (hexose and heptose region) was elucidated:



The cell wall lipopolysaccharides of Enterobacteriaceae consist of the inner lipid A portion, of the intermediate core oligosaccharide, and of the outer O-specific polysaccharide (1). While the lipid A structure appears to be the same in all Enterobacteriaceae, and while the structures of the O-specific side chains vary widely - giving rise to the large number of O serotypes -, the core oligosaccharides exhibit an intermediate variability: today, six different Enterobacteriaceae LPS* core types have been recognized (1). Of these, the primary structure of the E.coli R4 core (2,3) alone is still completely unknown. We therefor undertook its elucidation as follows:

MATERIALS AND METHODS

Media. Unless stated otherwise, $D_{1,5}$ medium (4) was used throughout.
Bacteria. Escherichia coli F2513, an acapsular and rough mutant of E. coli O14:K7(L):H⁻ with a complete R4 LPS core (3), was used. Deeper rough mutants of it were obtained with the help of rough specific phages, essentially as detailed previously (5,6).
Preparation of LPS and of LPS core oligosaccharides. E.coli F2513 and its mutants were grown in large batches as described by S. Schlecht (7). They were extracted by the method of C. Galanos et al. (8). The LPSs

*LPS = lipopolysaccharide

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thus obtained were degraded by heating in 1% aqueous acetic acid, and the core oligosaccharides were chromatographed through Biogel P2 after sedimentation of the insoluble lipid A (9). For dephosphorylation, the oligosaccharides were treated with 50% aqueous hydrofluoric acid (6, 10).

Constituent analyses of LPSs and of LPS core oligosaccharides. After hydrolysis (48 h at 100 °C in 0.1 N HCl), the aldoses were determined by g.l.c. of the alditol acetates (11; see also ref. 6). D-glucose and D-galactose were also estimated with fungal glucose oxidase (EC 1.1.1.4)(12), or with galactose dehydrogenase (EC 1.1.1.48) from Pseudomonas fluorescens (13). KDO* was determined by the thiobarbituric acid method (14), and phosphate according to O.H. Lowry et al. (15). Also following hydrolysis (18 h at 110, or 5 h at 100 °C in 4 N HCl, respectively), glucosamine, ethanolamine, and amino acids (protein) were determined with a DURRUM D-500 amino acid analyzer, and the fatty acids - extracted with petroleum ether and converted to the methyl esters with 1 N methanolic HCl (2 h at 100 °C) - by g.l.c. on a column of 2.5% Castowax on Chromosorb G (10); in the core oligosaccharides, however, the ethanolamine (free amino groups) was determined with ninhydrin (16). Nucleic acid was estimated from the absorption at 260 nm.

Molecular rotation. The molecular rotations of the LPS core oligosaccharides were calculated from the optical rotations as determined with a PERKIN ELMER model 141 polarimeter.

Methylation analyses of core oligosaccharides. The materials - before or after dephosphorylation (6,10) - were methylated with methyl sulfinyl carbanion in dimethyl sulfoxide (17,18). The methylated products were purified by passage over Sephadex LH20 and hydrolyzed, and the methylated monomers were identified by g.l.c./m.s. of the alditol acetates (or, occasionally, of the aldose acetates)(18,19; see also ref. 6). For localization of phosphate-containing substituents, the E.coli F2513 LPS core oligosaccharide was dephosphorylated (6,10) after methylation, and then remethylated with trideuteriomethyl iodide (20).

RESULTS

Escherichia coli F2513 is a rough strain (1,3), the cell wall LPS of which terminates in a complete R4 core oligosaccharide (2). To obtain partial R4 core oligosaccharides also (for the determination of the sequence of the constituent sugars, and of their anomeric configurations), deeper rough mutants (1) of E.coli F2513 were isolated with the help of rough specific phages (5,6). The cell wall LPSs of E.coli F2513 and of these mutants were then extracted (8), and the core oligosaccharides were prepared by mild acid hydrolysis (9). Both the complete LPSs and their isolated core oligosaccharides were subjected to quantitative constituent analyses (Tables 1 and 2).

Methylation/g.l.c./m.s. (18,19) of the R4 and R4 mutant LPS core oligosaccharides gave the results shown in Table 3; since the derivatives of phosphate-substituted heptoses are not detected by the method used, and since, on the other hand, dephosphorylation with hydrofluoric acid

*KDO = 3-deoxy-octulosonic acid

TABLE 1. Composition of Cell Wall Lipopolysaccharides (LPSs) from *Escherichia coli* R4 and *E.coli* R4 Mutants

Constituent	Weight percent[a] in LPS from *E.coli*:			
	F2513(R4)[b]	F2556[c]	F2566[c]	F257I[c]
D-Galactose[d]	9.5	6.0	4.1	trace
D-glucose[d]	6.7	5.8	8.3	trace
Heptose[e]	11.8	12.6	13.2	12.6
KDO[f]				
- determined	5.3	7.5	6.5	6.8
- calculated	13.4	14.2	15.0	21.5
D-Glucosamine	5.2	4.5	5.7	7.0
Phosphate	9.6	10.1	9.6	9.9
Ethanolamine	1.4	1.5	1.2	1.2
Lauric acid	2.6	2.8	2.9	4.5
Myristic acid	4.0	4.4	4.3	5.8
Palmitic acid	2.3	2.0	2.0	1.7
β-hydroxymyristic acid	17.2	19.3	19.1	27.0
Protein	0.2	0.4	0.4	0.1
Nucleic acid	<0.02	<0.02	0.04	0.09
Inorganic ash	13.5	14.9	13.5	14.7

[a] Weight percent anhydrosugar, or fatty acid minus H_2O. [b] Acapsular and rough mutant of *E.coli* 014:K7(L):H$^-$ with complete R4 core (2,3). [c] Deeper rough mutants of *E.coli* F2513. [d] About the same values were obtained enzymatically and by g.l.c. of the alditol acetates. [e] Alditol acetate co-chromatographing with authentic D-glycero-L-manno-heptitol acetate. The values of molecular rotation shown in Table 2, indicate L-glycero-D-manno-heptose (see also ref. 1). [f] KDO = 3-deoxy-octulosonic acid, probably 3-deoxy-D-manno-octulosonic acid (1); since the thiobarbituric acid method (14; after hydrolysis) does not recognize KDO residues substituted at C(4) or at C(5), another KDO value was calculated from the heptose value assuming three KDO per three heptose residues (see 1)(besides core oligosaccharide, mild acid hydrolysis of these LPSs yielded free KDO and KDO-ethanolamine).

(6,10) was found to cause loss of galactose also, both the native and the dephosphorylated oligosaccharides were methylated and the results combined (compare ref. 6). In addition to what is shown in Table 3, dephosphorylated LPS core oligosaccharide from *E.coli* F2571 was found to yield equimolar amounts of 2.3.4.6.7-Hep and 2.4.6.7-Hep.

TABLE 2. Composition and Molecular Rotation of Cell Wall LPS Core Oligosaccharides from *E.coli* R4 and *E.coli* R4 Mutants

	E.coli F2513(R4)	F2556	F2566	F2571
	Weight percent[a] (molar ratio) in LPS core oligosaccharide:			
D-Galactose	22.6(2.95)	17.0(2.08)	11.1(1.13)	-
D-Glucose	15.3(2.00)	16.4(2.00)	19.7(2.00)	0.5(0.02)
Heptose	28.5(3.14)	30.1(3.13)	27.8(2.42)	48.9(2.00)
Phosphate	13.2(3.26)	16.5(3.82)	16.2(3.10)	17.8(1.61)
Ethanolamine	4.9(1.76)	6.5(2.17)	5.2(1.44)	5.1(0.67)
	Molecular rotation of LPS core oligosaccharide:			
	+ 2039	+ 1895°	+ 1480°	+ 520°

[a] Weight percent anhydro sugar. In addition, the core oligosaccharides each contain one residue of reducing KDO (see 1).

For analysis of the anomeric linkage configurations, the molecular rotations of the core oligosaccharides were determined; the data are included in Table 2.

DISCUSSION

The results summarized in Tables 1 and 2 confirm the previous finding (3) that the *E.coli* R4 core oligosaccharide as obtained by mild acid hydrolysis of LPS (hexose and heptose regions) consists of three D-galactoses, two D-glucoses, three L-glycero-D-manno-heptoses, and one KDO (compare ref. 1). The data in Tables 1 and 2 further show that the *E. coli* R4 oligosaccharide is substituted by approximately two pyrophosphorylethanolamine residues, and that the oligosaccharides of the deeper rough R4 submutants *E.coli* F2556, F2566, and F2571 lack one galactose, two galactoses, or all hexoses and one heptose (see methylation results), respectively.

The positions of substitution, as well as the sequence of these constituents follow from the results of the methylation analyses (Table 3

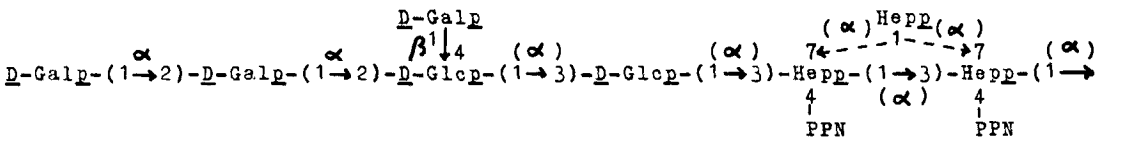
Table 3. Methylation/Gas-Liquid-Chromatography/Mass Spectrometry of *E.coli* R4 and *E.coli* R4 Mutant LPS Core Oligosaccharides

Alditol derivative[a]	T[b] Lit.[b]	T[b] found	Primary fragments found (m/e): 45	89	117	161	189	205	233	Approximate molar ratio[c] from LPS core in *E.coli*: F2513(R4)	F2556	F2566
2.3.4.6-Gal	1.25	1.25	+		+	+		+		1.4[d]	0.8	0.7[d]
2.4.6-Glc	1.95	1.97[e]	+		+	+			+	1.0[c]	0.9[c,e]	1.0[c]
3.4.6-Glc	1.98	1.95[e]	+			+	+	+	+	-	1.1[c,e]	-
2.3.4.6.7-Hep	2.13	2.10[f]	+	+	+	+		+		1.4	0.9	n.d.
2.3.6-Glc	2.50	2.40	+		+	+			+	-	-	1.1
3.4.6-Gal	2.50	2.50	+			+	+	+	+	1.1	1.0	-
2.4.6.7-Hep	4.40	4.50	+	+	+			+[g]	+[g]	1.0[c,g]	1.0[c]	n.d.
3.6-Glc	4.40	4.50	+				+		+	0.8[h]	-	-
2.4.6-Hep	13.0	13.8			+				+[g]	1.1[g]	1.3	n.d.

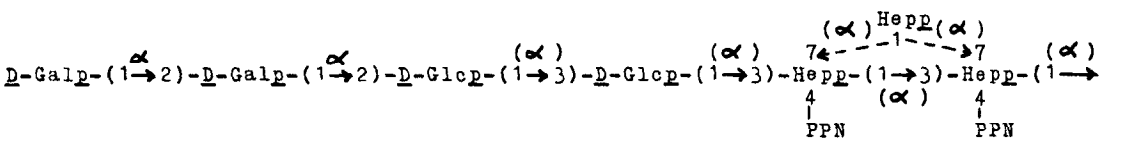
[a] 2.3.4.6-Gal = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol etc. [b] Retention time relative to 2.3.4.6-Glc (T = 1.00) and 2.3-Glc (T = 5.39) on ECNSS-M (6,10,18,19). [c] Peak ratio relative to 2.4.6-Glc = 1.0 (or to 2.4.6-Glc plus 3.4.6-Glc = 2.0 in F2556) for the hexitols (methylation before dephosphorylation), and relative to 2.4.6.7-Hep = 1.0 for the heptitols (methylation after dephosphorylation, see text). [d] Low yields of highly methylated hexitols are due to volatility. [e] 2.4.6-Glc and 3.4.6-Glc do not separate on ECNSS-M as the alditol acetates; they were separated as the aldose acetates, cochromatographing with standards (and giving the same mass spectra). [f] 2.3.4.6.7-Hep generally yields a second peak at T = 2.30, assumedly due to some decomposition during g.l.c. [g] Due to trideuteriomethylation at position 4, fragments m/e = 208 and 236 were obtained if methylated core oligosaccharide was subsequently dephosphorylated and then remethylated with trideuteriomethyl iodide. [h] Indistinguishable from 3.6-Gal; assignment concluded from the unequivocal assignment of three galactoses (and only one glucose) to other methylated alditol acetates.

TABLE 4. Cell Wall LPS Core Structures in *E.coli* R4 and *E.coli* R4 Mutants[a]

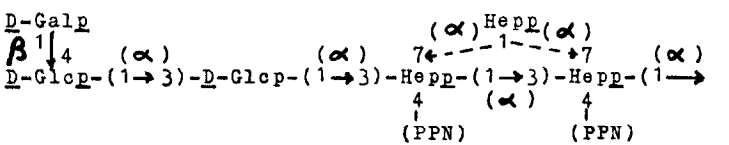
E.coli F2513(R4)



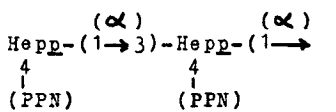
E.coli F2556



E.coli F2566



E.coli F2571



[a] D-Galp = D-galactopyranose, D-Glcp = D-glucopyranose, Hepp = heptopyranose (probably L-glycero-D-manno-heptopyranose; see ref. 1), PPN = pyrophosphorylethanolamine. In addition to what is shown, the core oligosaccharides each terminate in one reducing KDO residue (see ref. 1). The assignment of the branch to either of the chain heptoses is unknown. The anomeric configurations of the glucoses and the heptoses are tentative (see text). In *E.coli* F2566 and F2571, some PPN residues are lacking in addition to the sugar constituents (see Table 2).

and Results): if separate hexose and heptose regions are assumed, as well as chain heptose linkages through positions 3 (compare ref. 1), oligosaccharide F2566 must have the structure shown in Table 4 - or an alternative one with inverted locations of the 3- and 4-substituted glucoses. This latter possibility is excluded by the lack of a 4-substituted glucose in oligosaccharide F2556 (although both glucoses are present in it), a finding which also shows that the galactose unit present in oligosaccharide F2566 is absent in oligosaccharide F2556. On the basis of these conclusions, the additional methylation results in Table 3 directly yield the complete sequence and substitution patterns of oligosaccharides F2556 and F2513 (complete R4 core). The locations of the (approximately) two pyrophosphorylethanolamine residues on the chain heptoses follow from the results of methylation/dephosphorylation /trideuteriomethylation (see footnote "g" in Table 3)(20). The results do not allow an assignment of the branch heptose to the inner, or to the outer chain heptose.

α-configurations for the two (1→2)-linked galactoses follow from the difference in molecular rotation between oligosaccharides F2513 and F2566 = + 550° - see Table 2 and compare with the theoretical value of + 544° (21); and this conclusion is corroborated by the finding (data not shown) that α-galactosidase from green coffee beans (EC 3.2.1.22)(22) splits both galactoses off oligosaccharide F2556. In the same manner, the difference in molecular rotation between oligosaccharide F2513 and F2556 shows that the (1→4)-linked galactose is in the β-configuration, and the difference in molecular rotation between F2566 and F2571 can then be taken as an indication that the glucoses and the branch heptose may be α-configurated. Finally, the molecular rotation of the F2571 oligosaccharide indicates α-configurations for the chain heptoses also.

A comparison of the molecular rotations in Table 2 with those of *E. coli* C and *E.coli* C mutant LPS core oligosaccharides (6,20) - in which latter α-configurations of the two glucoses have been established by lectin precipitation (20) -, further substantiates the above assignment of anomeric linkages in the R4 core.

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