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LIPID A COMPONENT OF SALMONELLA TYPHIMURIUM CARRYING THE DEREPRESSED Col Iб PLASMIDS

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The structure of the lipid A from S. typhimurium harboring the derepressed plasmids Col Iб is very similar: i, 1,4-bis-phosphorylated-β-1′,6-linked glucosamine disaccharide forms a backbone of the lipid; ii, lipid preparations contain four residues of 3-hydroxytetradecanoic acid at positions C3, C3′ and the amide linked at C2, C2′ and two free hydroxyl groups at positions C4 and C6′. Differences concern: i, substitution of phosphoryl groups by 4-amino-4-deoxy-L-arabinopyranose and phosphorylethanolamine in S. typhimurium with Col Iб plasmids; ii, the degree of acylation of hydroxyl groups of 3-hydroxytetradecanoic acid by myristic, lauric and palmitic acids; iii, presence of tridecanoic acid bound to hydroxyl of 3-hydroxytetradecanate residue in S. typhimurium with Col IбΔrд2 plasmid.

Lipopolysaccharides from the plasmid mutant strains express several times higher lethal toxicity in chick embryos compared to lipopolysaccharides from the strain with the wild type Col Iб.

Endotoxins, the surface components of the outer membrane of gram-negative bacteria, play an important role in the interaction between bacteria and higher organisms. Chemically, they are lipopolysaccharides (LPS)1 consisting of a polysaccharide and lipid A [1]. In all Enterobacteriaceae lipid A has a similar structure, a detailed analysis of this compound from Salmonella (Scheme 1) revealed the presence of β-D-glucosaminyl-1′,6-D-glucosamine disaccharide, phosphorylated at positions C1 and C4′. To this hydrophilic backbone is linked by ester and amide bonds up to seven hydroxylated and nonhydroxylated saturated fatty acid residues. The biological activities of endotoxins are mediated mainly by lipid A and appear to depend on its

1 Abbreviations: LPS, lipopolysaccharide; h.v.p.e., high voltage paper electrophoresis; P, phosphate; GlcN, 2-Amino-2-deoxy-D-glucopyranose (D-glucosamine); GlcN-6-P, 2-Amino-2-deoxy-D-glucopyranose-6-phosphate (D-glucosamine-6-phosphate); GlcNAc, 2-Acetamido-2-deoxy-D-glucopyranose (N-acetyl-D-glucosamine); 4-AraN, 4-Amino-4-deoxy-L-arabinopyranose; d-Ara, d-arabinofuranose; EtN, ethanolamine; PEtN, phosphorylethanolamine; dOc1A, 2-keto-3-deoxy-D-manno-octonate (KDO).
Scheme 1. Proposed chemical structure of lipid A of Salmonella minnesota [4]. Dotted lines indicate incomplete substitution. Numbers in circles indicate the number of carbon atoms in the acyl chains.

peculiar conformation, determined by both, its supramolecular structure and fluidity of its acyl chains [1].

The three different structural regions of LPS (O-antigen chains, core oligosaccharide and the lipid A) are independently synthesized by different biosynthetic pathways regulated by three gene clusters localised on the chromosome. However, in some bacteria these basic structures can be modified by extrachromosomal elements, such as bacteriophage genomes or plasmids [6].

Some virulence plasmids required for the expression of the O-antigenic specific polysaccharides are known to confer a wide variety of phenotypic modifications and genetic flexibility upon their hosts [7-10]. Among these plasmids Col Ib of average \( M_r \) 61 500 000 are relatively large and may well carry genes for other characters [11]. In our previous studies we have found the pleiotropic effects caused by Col Ib plasmids derepressed in the genes coding for colicin synthesis and the ability of self transfer [12]. In strain S. typhimurium Rc902 infected with Col Ibdrd2 mutant, derepressed in colicin production, composition of LPS [13] was altered, whereas bacteria carrying Col Ibdrd7 plasmid derepressed in transfer, exhibited a marked decrease in the content of all O-antigen chain sugars [14]. The strains of S. typhimurium Rc902 carrying the wild type Col Ib or the revertants of drd mutants to the wild type showed no differences in the sugar composition of LPS.
In this study the structure of the lipid A region of LPS from *S. typhimurium* Rc902 strains harboring the derepressed Col Ib mutants has been established and compared to lipid A from strain with the parental plasmid. In our comparative studies we have focused the attention especially on fatty acyl residues and the phosphodiester group.

**EXPERIMENTAL**

*Strains.* The colicinogenic strain *S. typhimurium* Rc902 cysD36 Col Ib was kindly provided by R. C. Clowes. The mutants *S. typhimurium* Rc902 Col Ibdrd2 and *S. typhimurium* Rc902 Col Ibdrd7 derived from strain Rc902 Col Ib were derepressed for colicin production or in the plasmid transfer ability, respectively [12].

*Isolation of LPS and lipid A.* For the isolation of LPS the cells were grown to the stationary phase, in shake cultures on nutrient broth supplemented with 0.5% glucose at 37°C. LPS's of S type were isolated and purified by the hot phenol-water procedure [15], and that of R type with phenol-chloroform-petroleum ether [16]. Lipids A were prepared by boiling LPS with 1% acetic acid for 2 h.

*Isolation of the lipid A backbone.* The isolation and chemical analysis of the lipid A sugar backbone was carried out according to Hase & Rietsechel [2].

*Methylation.* The backbone disaccharide was methylated as described by Hakomori [17]. Direct methylation of free and dephosphorylated lipid A was done according to Sidorczyk et al. [18]. Lipid A was dephosphorylated by 48% aqueous hydrofluoric acid [19].

*Fatty acid analyses.* The determination of total as well as ester and amide linked fatty acids was performed as described by Rietsechel et al. [20]. Double bonds in unsaturated fatty acids were located according to Harvey [21]. The content of 3-acyloxyacyl residues was determined as described by Wollenweber et al. [3].

*Other analytical methods.* The procedure of Lowry et al. [22] was used for determination of total phosphate, and the Morgan-Eelson reagent in the modification of Strominger et al. [23] for glucosamine.

H.v.p.e. was carried out using the buffer system pyridine/acetic acid/water (10:4:86, by vol.) at pH 5.3. Electrophoreograms were stained with ninhydrin, alkaline silver nitrate or the modified ammonium molybdate reagent [24].

G.l.c. was performed on GCHF Giede-18.3 gas chromatograph, equipped with the following columns: (A) a glass column (0.25 × 200 cm) packed with 3% ECNSS-M on 100-120 mesh Gas Chrom Q at 178°C or 190°C, (B) a glass column (0.25 × 200 cm) packed with 3% or 10% SE-30 on 100-120 mesh Gas Chrom Q at 185°C, and (C) a glass column (0.30 × 180 cm) packed with 15% EGSS-X on 100-120 mesh Gas Chrom P at 170°C.
G.c.-m.s. was done with a Finnigan MAT1020B automatic system on a fused capillary column (0.25 mm × 25 m) coated with OV-101, temperature programming from 170°C to 250°C at 2°C min⁻¹ or from 100°C to 280°C at 5°C min⁻¹.

³¹P-n.m.r. spectra were recorded on a Brucker WM300 apparatus at 121.51 Hz using 85% phosphoric acid as an external standard. Samples were dissolved in ³¹H₂O containing 5 mM EDTA and 2% sodium deoxycholate.

Lethality test in chick embryos. The fifty percent chick embryo lethal dose CELD₅₀ was estimated according to Konno [25] with the following modifications: the LPS samples (of five replicates each) of 50; 5; 0.5; 0.05; 0.005; and 0.0005 μg (in 2 ml of NaCl/Pi buffer) per embryo, were injected intraamniotically. The results were estimated after 38 h incubation at 37°C.

RESULTS

Comparison of lipid A preparation from S. typhimurium with the wild and mutant plasmids

By mild acid hydrolysis of LPS from S. typhimurium Re902 containing plasmids Col Ib, Col Ibdrd2 and Col Ibdrd7 lipid A preparations were obtained with the following yields: 30.3%; 23.5 and 44.5% (w/w), respectively. The last value indicated the rough type of LPS from this strain. The molar ratio of GlcN to P in the O-deacylated LPS preparations i.e., in LPS-OH was approx. 1.0 in the preparations from the strains with wild type plasmid and with Col Ibdrd7, whereas in LPS-OH from the strain with Col Ibdrd2 it was 1.3 (Table 1). This last value suggests more intensive release of the ester bound phosphate diester (Table 1). A similar release of the ester bound P substituent, by β-elimination was described by Sidorchzyk et al. [18]. This suggestion was further confirmed by the finding that the content of GlcN-6-P in LPS-OH from S. typhimurium Re902 Col Ibdrd2 was lower than in the corresponding preparations from the two remaining strains.

Mild acid hydrolysis of LPS-OH resulted in formation of lipid A-OH. A positive direct Morgan-Elson reaction in the lipid A-OH from each strain examined, and the failure of this reaction in the reduced lipid A-OH indicated the presence of reducing GlcN residue.

Hydrazinolysis of lipid A-OH_red preparations yielded a phosphate free "pseudooligosaccharide", which was isolated by preparative electrophoresis (mGlcN 1.14) at pH 5.3. The material, after N-acetylation and permethylation was characterized by g.c.-m.s. as glucosaminyl-β-1,6-glucosaminitol. This was based on the retention time on SE-30 column (cf. Experimental, column B) and on its characteristic mass fragmentation pattern, described elsewhere [26]. The configuration β of the 1',6 linkage was additionally confirmed by positive immunoprecipitation with type II lectin from Ulex europaeus (Sigma, of specific affinity to β-glycosidically linked polymers of GlcNAc) and N-acetylated
Table 1

The content of glucosamine and phosphate in the degradation products of LPS leading to the lipid A backbone

<table>
<thead>
<tr>
<th>S. typhimurium Re902 with plasmid</th>
<th>Preparation</th>
<th>Yield</th>
<th>µmol/mg of preparation</th>
<th>GLcN</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>direct after hydrolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col 1b</td>
<td>LPS</td>
<td>100</td>
<td>traces</td>
<td>0.426</td>
<td>0.571</td>
</tr>
<tr>
<td></td>
<td>LPS-OH</td>
<td>84</td>
<td>traces</td>
<td>0.660</td>
<td>0.637</td>
</tr>
<tr>
<td></td>
<td>lipid A-OH</td>
<td>20</td>
<td>0.431</td>
<td>0.856</td>
<td>0.416</td>
</tr>
<tr>
<td></td>
<td>lipid A-OH&lt;sub&gt;red&lt;/sub&gt;</td>
<td>41</td>
<td>0.000</td>
<td>0.432</td>
<td>0.374</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>100</td>
<td>0.010</td>
<td>0.492</td>
<td>0.677</td>
</tr>
<tr>
<td></td>
<td>LPS-OH</td>
<td>85</td>
<td>traces</td>
<td>0.672*</td>
<td>0.522</td>
</tr>
<tr>
<td></td>
<td>lipid A-OH</td>
<td>23</td>
<td>0.477</td>
<td>0.882</td>
<td>0.243</td>
</tr>
<tr>
<td></td>
<td>lipid A-OH&lt;sub&gt;red&lt;/sub&gt;</td>
<td>34</td>
<td>0.000</td>
<td>0.454</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>100</td>
<td>traces</td>
<td>0.640</td>
<td>0.776</td>
</tr>
<tr>
<td></td>
<td>LPS-OH</td>
<td>70</td>
<td>traces</td>
<td>0.679*</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>lipid A-OH</td>
<td>41</td>
<td>0.842</td>
<td>0.928</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>lipid A-OH&lt;sub&gt;red&lt;/sub&gt;</td>
<td>33</td>
<td>0.000</td>
<td>0.473</td>
<td>0.411</td>
</tr>
</tbody>
</table>

* The molar ratio of GLcN/GLcN-6-P determined by amino-acid analysis was 5.8 in the LPS-OH from the strains with plasmid Col 1b and Col 1bdr<sub>d2</sub>, whereas in that with Col 1bdr<sub>d7</sub> it was 1.7.

derivatives of lipid A backbone disaccharides. This central disaccharide was identical in lipids A from all the strains examined.

Localization of unsubstituted hydroxyl groups in the backbone disaccharide was carried out by silica gel-catalysed methylation of free lipids A. The resulting material yielded, after hydrolysis, 4-O-methyl-1,3,5,6-tetra-O-acetyl-2-deoxy-2(N-acetamido)glucitol, 6-O-methyl-1,3,4,5-tetra-O-acetyl-2-deoxy-2(N-acetamido)glucitol and peracetylated glucosaminitol. Additionally a small peak of 3-O-methyl derivative of glucosaminitol was detected. The presence of peracetylated glucosaminitol is due to undermethylation, as observed earlier [27]. Also it should be concluded that 6-O-methylated derivative formed originated from the non-reducing GLcN since 4-O-methyl derivative should result from the reducing GLcN, and 4,6-di-O-methyl was not found. This also indicates the site of attachment of dOclA (KDO) at 6' position of lipid A.

The same procedure applied to the dephosphorylated lipid A preparations revealed a new derivative i.e., 4,6-di-O-methyl-1,3,5-tri-O-acetyl-2-deoxy-2(N-acetamido)glucitol, which indicates C4' position of phosphate at the non-reducing GLcN.

The substituents of phosphoryl residues in lipids A

The presence of 4-amino-4-deoxy-L-arabinose in all examined strains was suggested because of characteristic orange colour with ninhydrin and m<sub>GLcN</sub> 1.20 identical with that of product released from LPS of *Proteus mirabilis* R<sub>45</sub>.
on mild acid hydrolysis (0.5 M HCl, 16 h at 37°C). Complete substitution of the ester linked P by 4-AraN was documented in lipid A of Proteus mirabilis. However, 4-AraN was not found on mild alkali treatment of LPS (0.20 M NaOH, 1 h at 100°C) although a spot, which was positive with ninhydrin and ammonium molybdate appeared at start of electrophoregram. These results indicated an acid labile linkage of 4-AraN ester bound with P residue in the lipid A moiety, as it was demonstrated previously by other authors [28]. The identification of this component as alditol acetate by g.c.-m.s. was finally confirmed after acid hydrolysis of N-acetylated LPS preparations. The fragmentation pattern of the product showed fragments comparable with those earlier described [18]. On the basis of previously published data [1, 4, 29] we assume l-configuration for 4-amino pentose. Quantitation of 4-AraN (Table 2)

**Table 2**
The content of the non-fatty acid components (μmol/mg of lipid A) in the lipid A preparations from *S. typhimurium* Rc902

<table>
<thead>
<tr>
<th><em>S. typhimurium</em> Rc902 with plasmid:</th>
<th>GlcN</th>
<th>P</th>
<th>PEtN*</th>
<th>EtN*</th>
<th>4-AraN**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col Ib</td>
<td>0.746</td>
<td>0.740</td>
<td>0.08</td>
<td>0.12</td>
<td>0.16</td>
</tr>
<tr>
<td>Col Ib*rd2</td>
<td>0.765</td>
<td>0.785</td>
<td>0.03</td>
<td>0.05</td>
<td>0.21</td>
</tr>
<tr>
<td>Col Ib*rd7</td>
<td>0.806</td>
<td>0.770</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Estimated by the amino-acid analyser
** Determined by g.l.c. in LPS preparations

demonstrated that its content in the LPS from the strain with parental plasmid and from the strain with Col *Ib*rd2 (0.05 μmol/mg of LPS), was similar whereas in the LPS of *S. typhimurium* Rc902 Col *Ib*rd7 it was lower (0.03 μmol/mg of LPS). Although the results obtained by g.l.c. are semi-quantitative, calculated approximately 4-AraN content in lipid A preparations (Table 2) was in agreement with the suggestions drawn from the data given in Table 1 of the relatively higher content of 4-AraN in the lipid A from the strain with Col *Ib*rd2 than in to the lipids A from the two remaining strains.

The identification of another substituent linked to the glycosidically bound P residue (cf. Table 1) was performed by h.v.p.e. Among the products released after 20 min mild acid hydrolysis (0.05 M HCl at 100°C) the following compounds were identified; P₁ and the unknown compound migrating toward anode, at about twice as low rate as P₁ and migrating toward the cathode PEtN (m<sub>GlcN</sub> 0.08), 4-AraN (m<sub>GlcN</sub> 1.20) and traces of EtN (m<sub>GlcN</sub> 2.20). On prolongation of hydrolysis to 60 min the spot disappeared with a simultaneous increase intensity of EtN and PEtN spots. These results suggested the linkage of some PEtN residues to the glycosidically bound P of the reducing GlcN. Quantitative determination of the whole substituent, i.e. PPtEtN, is difficult
Fig. 1. $^{31}$P-n.m.r. spectra of free lipid A from *S. typhimurium* Rc902 Col 1bdrd2 at pH 7.4 (A) and after overnight incubation at room temperature, at pH 10.5 (B)
because of its extreme lability. The estimation of EtN and PEtN cleaved revealed a lower degree of glycosidically linked P by PEtN in lipids A from the strains with the derepressed plasmids Col Ib than in the strain with the parental plasmid (Table 2).

Localization of P residues in free lipid A from the strains with mutant plasmids was confirmed by $^{31}$P-n.m.r. The preparation of S. typhimurium Rc902 Col Ibdrd2 showed three dominating signals at: 1.21; 1.60 and 4.09 ppm at neutral pH value (Fig. 1A), shifted downfield to 2.43; 4.32 and 4.81 ppm, respectively, on rising pH to 10.5 (Fig. 1B) due to the pH-dependent change in the phosphate ionization of phosphomonoesters [5]. On the basis of these data and the chemical shift range observed by other authors [5, 29, 30, 31] we can assume that the main peaks at 4.32 and 4.81 ppm corresponded to the ester linked $\beta$-4'-phosphomonoester signals and those at 2.53 and 2.43 ppm to the $\alpha$-glycosidic phosphomonoester (Fig. 1B). The multiplicity of signals of ester and glycosidically linked phosphomonoesters is due to the heterogeneity of the substitution of hydroxy fatty acids: even larger changes in chemical shift are known to occur for the C4' phosphomonoester group [5, 31]. Thus the divergence of the signals at 4.81 and 4.32 ppm could correspond to the phosphorylated GlcN residue with 3-hydroxy or 3-acyloxy fatty acid at the C3' position, respectively. In this lipid A the only pH-independent signal was a small one at 0.75 ppm which could be ascribed to the C4' phosphate diester according to the data published [5, 30]. In the range of pyrophosphate signals i.e. 10-12 ppm [5, 31] the double signals of very low intensity were observed at 10.36; 10.51 ppm and 10.83; 10.91 ppm, while no signals at around 5.0 ppm were detected (Fig. 1A). This could indicate the presence of substituted (by EtN) glycosidically linked pyrophosphate residue in lipid A of S. typhimurium Rc902 Col Ibdrd2. The low content of this substituent detected in free lipid A is due to its substantial loss during the release of lipid A from LPS. $^{31}$P-n.m.r. spectrum of free lipid A of S. typhimurium Rc902 Col Ibdrd7 was very similar to that carrying Col Ibdrd2 (not shown). Small differences were observed only in the proportion of two C4' phosphomonoester signals, which indicates a lower degree of acylation of C3' linked hydroxy fatty acid residue in this lipid A. The signal of the ester linked phosphodiester residue was very weak but those of glycosidically bound pyrophosphodiesters were higher compared to the corresponding signals in lipid A from S. typhimurium Rc902 Col Ibdrd2. These results confirmed slight differences in substitutions of both phosphate groups of the backbone disaccharides in lipid A from the plasmid mutant strains (Table 2).

The acyl residues

By means of g.c.-m.s. analysis of fatty acids released from free lipids A in all preparations from the bacteria grown at 37°C the following major fatty acids were detected: dodecanoic (12:0), tetradecanoic (14:0), hexadecanoic (16:0)
and 3-hydroxymyristic (3-OH-14:0). Other fatty acids, hexadecenoic (16:1), 2-hydroxymyristic (2-OH-14:0), and 3-hydroxyauric (3-OH-12:0) were present in lower amounts. Tridecanoic acid (13:0) has been found only in lipid A of *S. typhimurium* Rc902 Col Ibdrd2. In addition to the above mentioned fatty acids, all preparations contained trace amounts (below a few nanomoles per mg of lipid A) of decanoic (10:0), branched undecanoic (11:0br), tetradecenoic (14:1), branched pentadecanoic (15:0br), octadecenoic (18:1) and 3-hydroxydecanoic (3-OH-10:0) acid. Determination of fatty acids in the LPS preparations isolated from bacteria cultivated at 25°C revealed similar differences in the total fatty acid content i.e. the amount of 14:1 and particularly of 16:1 fatty acids increased, whereas that of 12:0 and 16:0 decreased. The most significant temperature dependent changes in fatty acids content were found in the LPS from *S. typhimurium* Rc902 Col Ibdrd2. In LPS isolated from the culture grown at lower temperature almost quantitative replacement of 16:0 and 13:0 fatty acids by 16:1 one was observed. G.c.-m.s. of this fatty acid in the form of 3-picolinyl ester revealed an intense pair of fragments at m/e 234 and 260 together with a low intensity pair at m/e 274 and 288, which indicates 9,10 position of the double bond (Fig. 2).

It was established that, in all lipids A examined, 3-OH-14:0 together with small amounts of 3-OH-12:0 fatty acids were amide bound. On alkaline methanolsysis of lipid A preparations some 3-methoxy derivative of 3-OH-14:0 was detected besides the methyl esters of other fatty acids bound by ester linkages. Thus it should be concluded that the methoxy derivative was formed by β-elimination from ester linked 3-OH-14:0 with acylated hydroxyl group. The results of identification of the acyl or acyloxyacyl residues specifically

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Fig. 2. Mass spectrum of 3-picolinyl ester of 9,10-hexadecenoic acid isolated from LPS of *S. typhimurium* Rc902 Col Ibdrd2 bacteria cultivated at 25°C
released from the ester or amide linkages in lipid A preparations are presented in Table 3. Basing on the m/e fragmentation pattern obtained in electron impact and chemically ionization mass spectra, the two major amide-bound acyloxyacyl residues were identified in all examined strains namely: 3-dodecanoyloxytetradecanoyl and 3-hexadecanoyloxytetradecanoyl together with trace amounts of 3-dodecanoyloxydodecanoyl and 3-decanoyloxytetradecanoyl. Additionally in lipid A from *S. typhimurium* Rc902 Col *Ibдрd2* the amide bound 3-tridecanoyloxytetradecanoyl (Fig. 3) and traces of 3-undecanoyloxytetradecanoyl groups were identified. All lipid A preparations contained ester linked 3-tetradecanoyloxytetradecanoic acid and very low amounts of 3-(2)-hydroxytetradecanoyloxytetradecanoic acid.

![Chemical structure](image1)

**Fig. 3.** Mass spectrum of the tridecanoyloxytetradecanoic acid methyl ester obtained from the lipid A of *S. typhimurium* Rc902 Col *Ibдрd2*
Table 3
The molar ratio of ester and amide bound fatty acids and acyloxyacyl residues in lipid A from S. typhimurium Rc902

<table>
<thead>
<tr>
<th>Acyl residue</th>
<th>S. typhimurium Rc902 with plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col Ib</td>
</tr>
<tr>
<td>OAc</td>
<td></td>
</tr>
<tr>
<td>3-OH(14:0)</td>
<td>0.79</td>
</tr>
<tr>
<td>3-OH(14:0)</td>
<td>0.08</td>
</tr>
<tr>
<td>3-OH(14:0)</td>
<td>0.84</td>
</tr>
<tr>
<td>OAc</td>
<td></td>
</tr>
<tr>
<td>3-OH(12:0)</td>
<td>0.65</td>
</tr>
<tr>
<td>3-OH(13:0)</td>
<td>0.00</td>
</tr>
<tr>
<td>3-OH(16:0)</td>
<td>0.57</td>
</tr>
<tr>
<td>3-OH(12:0)*</td>
<td>0.01</td>
</tr>
<tr>
<td>3-OH(14:0)</td>
<td>0.43</td>
</tr>
<tr>
<td>Total fatty acids**</td>
<td>5.53</td>
</tr>
</tbody>
</table>

* 3-OH(12:0) occurred in the fraction of the amide linked acyloxyacyl residues as 3-OH(12:0)-12:0 giving a peak comigrating with 3-OH(16:0)-16:0.
** Determined by acid methanolysis of free lipid A preparations.

The results presented in Table 3 demonstrate a high similarity in the substitution pattern of the ester and amide linked 3-OH(14:0) residues in lipids A from the strain with the parental plasmid Col Ib and that with plasmid Col lbdrd7. Some differences occur, however, in lipid A from another plasmid mutant strain in which ester linked 3-OH(14:0) residues are almost totally acylated whereas the amide linked the 3-OH(14:0) are substituted by 16:0 lower by half amount and are additionally acylated by 13:0.

Biological response to mutation of Col Ib plasmids

Lethal toxicity of LPS was determined in chick embryos (Table 4). The most active was LPS from S. typhimurium Rc902 Col lbdrd7. However, when the activity is referred to lipid A, the preparations from both plasmid mutant strains appear to be approximately five times more effective than LPS from bacteria with the wild type Col Ib. It seems noteworthy that the most striking morphological effects were caused by LPS from S. typhimurium Rc902 Col lbdrd2.

Table 4
Toxicity of LPS from S. typhimurium Rc902 for 9-day-old chick embryos (CELD<sub>50</sub>).

<table>
<thead>
<tr>
<th>S. typhimurium Rc902 with plasmid</th>
<th>Number of embryos dead out of five at the LPS concentration (µg/per embryo):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Col Ib</td>
<td>3</td>
</tr>
<tr>
<td>Col lbdrd2</td>
<td>4</td>
</tr>
<tr>
<td>Col lbdrd7</td>
<td>5</td>
</tr>
</tbody>
</table>
DISCUSSION

Recently it has become evident that LPS structure is influenced by plasmids in \textit{Shigella} \cite{7}, \textit{E. coli} \cite{9} and \textit{Salmonella} \cite{8, 10}. Therefore the plasmid-mediated alteration of LPS could be a common phenomenon among enteric bacterial pathogens.

The results of previous investigations demonstrated the relationship between the structure of \textit{O}-specific part of LPS and the presence of the derepressed plasmids Col Ib in \textit{S. typhimurium} Rc902 \cite{13, 14}. The results of the structural studies on lipid A components of LPS from two \textit{S. typhimurium} Rc902 strains harboring the derepressed plasmids Col Ib\textit{drd2} and Col Ib\textit{drd7} points to their structural similarity with the lipid A from the strain with parental plasmid. The lipids A isolated from the strains studied exhibited a common backbone, namely 1,4'-bis-phosphorylated-\textit{β}-1',6-linked glucosamine disaccharide. Both phosphate groups carry the same substituents i.e. 4-AraN bound to C4' phosphate and PEtN bound to glycosidic phosphate, although in non-stoichiometric amounts. The differences in the degree of phosphate group substitution might partly result from degradation of these high acid labile components during lipid A isolation \cite{31}. On the other hand the phosphate-containing lipids A are known to undergo modification when adopted to certain environmental changes, by a partial or total substitution of the backbone phosphates by cationic or neutral substituents (4-AraN, PEtN, D-GlcN or D-Ara\textit{p}) \cite{28}.

Some variability was observed in fatty acid composition due to acylation of the residues of the ester and amide bound 3-hydroxytetradecanoic acid. These differences were only quantitative in lipids A from \textit{S. typhimurium} Rc902 Col Ib\textit{drd7} and \textit{S. typhimurium} with the wild type Col Ib. In the strain with Col Ib\textit{drd2}, tridecanoic acid was found which acylate a part of the amide linked 3-OH-14:0 residues. Further studies are required to establish the location of the amide linked acyloxyacyl residues in these lipids. However, the results obtained by other authors indicate that 3-O-acylating fatty acids of shorter chain length acylate 3-OH-14:0 amide group bound to non-reducing GlcN residue, while 16:0 substitutes (in non-stoichiometric amounts) the 3-hydroxyacyl group which is attached to reducing GlcN residue. The finding of lower quantities of 3-O (16:0)-14:0 residue than of 3-O (12:0)-14:0 in all strains studied and particularly in lipid A of \textit{S. typhimurium} Rc902 Col Ib\textit{drd2}, is only partly due to lower liberation of 3-O (16:0)-14:0 during methylation of lipid A \cite{19}. As it was demonstrated in the studies on lipid Y precursor of lipid A and the palmitate-containing species, this substituent more likely results from side reactions, since palmitate is practically absent in mature lipid A of Re mutant of \textit{S. typhimurium} or \textit{E. coli} K12 \cite{31}. When growth temperature was decreased the strain \textit{S. typhimurium} Rc902 Col Ib\textit{drd2} exhibited more evident changes in fatty acids composition. Incorporation of 4\textsuperscript{o}-cis-hexadecenoic acid
into lipid A was also demonstrated in *E. coli*, *P. mirabilis* and *S. minnesota*; in lipids A isolated from these bacteria cultivated at 12°C an almost quantitatively replacement takes place of saturated fatty acid by unsaturated fatty acid in to the non-reducing GlcN residue [1, 3, 19]. The preparations of LPS from both plasmid mutants exhibited higher toxicity for chick embryos than LPS that from the parental strain despite rather small structural differences in their lipids A. The higher toxicity of LPS from *S. typhimurium* Rc902 Col lbadr1l7 could be partly explained by a higher share of lipid A part in its LPS molecule.

On the other hand there is some evidence that different LPS preparations isolated from the same batches of bacteria often have a slightly different endotoxic potency due to the commonly occurring heterogeneity of lipid A differing in fatty acid pattern and the polar head groups (PEtN, AraN) of the backbone phosphates. Although the last compounds do not contribute to the endotoxic properties of lipid A they may exhibit an indirect effect by increasing solubility of lipid A [1, 28].

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**REFERENCES**


