CHARACTERIZATION OF THE LIPOPOLYSACCHARIDE FROM THE POLYMYXIN-RESISTANT *pmrA* MUTANTS OF *SALMONELLA TYPHIMURIUM*

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1. Introduction

Lipopolysaccharide (LPS, endotoxin) is a major constituent of the outer membrane (OM) of Gram-negative bacteria and is essential for the proper assembly, organization, and function of this membrane [1,2]. The complex chemical structure of LPS involves the O-specific chain, the core oligosaccharide, and the lipid A component and has been largely elucidated in many Gram-negative bacteria, mainly enteric bacteria (reviewed in [3–5]).

Lipopolysaccharides, e.g., those of *Salmonella*, exhibit structural heterogeneity of thus far unknown significance. One striking example is the wide heterogeneity in the length of the O-specific chain in smooth bacteria [6–9]. Another example is the partial substitution of phosphate residues located in the core oligosaccharide and the lipid A component by residues such as phosphorylethanolamine (PEtn) and 4-amino-4-deoxy-L-arabinose (4-ARAN) [10,11].

We have isolated *S. typhimurium* mutants (*pmrA* mutants) which have outer membranes with increased resistance to polymyxin, a basic, amphipathic antibiotic as well as to other cationic agents and Tris–EDTA ([12–14] and M. V., submitted). Here we show that these *pmrA* mutants represent a novel type of mutation affecting the lipid A structure: the ester-linked phosphate in their lipid A is almost completely substituted with 4-ARAN. Their LPS has also an increased content of ethanolamine.

2. Materials and methods

2.1. *Bacterial strains and LPS*

SH7585 (*pmrA*[+]) and SH7580 (*pmrA*) are *S. typhimurium* LT2 strains with the *galE* mutation from SH6749 [6]. The *Proteus mirabilis* Re strain R45 was a kind gift of Professor K. Kotelko (Lodz). The relevant genotypes of the other strains are found in table 1. The bacteria were grown overnight at 37°C on glucose-enriched nutrient agar (slightly modified after [16] per liter): Lab-Lemco broth (Difco), 20 g; yeast extract (Difco), 5 g; glucose, 3 g; $Na_2HPO_4 \cdot 12\,H_2O$, 2.3 g; agar, 15 g. LPS was extracted by the method in [17].

2.2. *Polymyxin binding to LPS*

LPS, 80 nmol (sodium salt of the electrodialyzed [18] LPS) was incubated with 200 nmol polymyxin B sulfate in 3 ml 0.07 M potassium phosphate (pH 7.2) for 30 min at 37°C. The precipitate formed was pelleted at 20 000 × *g* for 20 min and resuspended in 300 μl of 10% SDS. Of the LPS, 80–97% (based on dOclA content) was found in this preparation. The polymyxin B content of the precipitate was calculated from the number of free amino groups, corrected for the number of LPS amino groups.

2.3. *Chromatographic methods*

Carbohydrate analyses using gas–liquid chromatography were done as in [19,20]. Thin-layer chromatography of the ultrafiltrate (Millipore Immersible CX unit, cutoff 10 000 M_r) from alkali-treated LPS was carried out on silica gel plates (60, Merck, Darmstadt) using isobutanol/water (9:1, v/v, solvent A) or butanol/pyridine/water (6:4:3, v/v/v, solvent B). Phosphorus was detected using Hanes-Isherwoods reagent [21]. For fatty acid analysis, LPS samples were methanolysed (methanol/concentrated sulfuric acid, 94:6, v/v; 7 h; 70°C) in the presence of an internal standard

(*n*-heptadecanoic acid). The methyl esters were analyzed with a gas–liquid chromatograph (Carlo Erba Fractovap 2150, Milano) operating at 170°C.

2.4. *Other analytical methods*

Total phosphorus was determined according to [22], dOclA with the thiobarbituric acid method [23], amino-groups with ninhydrin [24], heptose with H_2SO_4/cysteine [25], and glucosamine after acid hydrolysis (4 N HCl, 100°C, 15 h) using the Morgan-Elson reaction [26]. An automatic amino acid analyzer (Durrum D 500) was used for the estimation of ethanolamine containing compounds and 4-ARAN. Phosphorylethanolamine gave a single peak eluting with glucosamine phosphate; the amount of glucosamine phosphate was calculated as the difference between the amounts of glucosamine found with amino acid analyzer and in Morgan-Elson reaction.

For 4-ARAN analysis [27] LPS was subjected to mild acid hydrolysis (0.5–3.0 mg LPS in 880 μl 0.1 N HCl, 100°C, 20 min). The hydrolysate was neutralized with NaOH and centrifuged at 8000 × *g*. Samples of the supernatant gave a color reaction with diphenylamine [28] with the absorption maximum at 555 nm. Within the absorbances used (0.1–0.4) the absorbance *vs* amino-sugar concentration plot was linear. dOclA (also after similar acid hydrolysis) gave no color reaction.

2.5. *Alkali-treatment of LPS and isolation of free lipid A*

LPS (3 mg) was treated in 720 μl 0.17 M NaOH (100°C, 1h) whereafter the mixture was neutralized with 1 N HCl. For subsequent isolation of free lipid A [29], 160 μl 1 N HCl was added and the mixture further incubated (100°C, 1 h). After addition of water (5 ml) and chloroform/methanol (2:1, v/v; 1 ml) and after vigorous shaking, the chloroform phase, together with the floating pellicle in the interphase, was washed 2 times with water (5 ml) and evaporated into dryness. The free lipid A was dissolved in chloroform/methanol/water (140:45:7, v/v/v).

3. Results

3.1. *Polymyxin binding to LPS*

The binding values for the parent *rfaJ* strains approached 1 mol PM/mol LPS (table 1) consistent with our results using a less direct method [13], and with the value in [30] for a comparable *Salmonella* strain. The 'heptoseless' RfaE LPS bound somewhat less PM (0.68 mol/mol LPS) than the more complete RfaJ LPSs (~0.8 mol/mol LPS). This could be due to the lack of potential PM-binding groups, viz. the phosphate residues linked to heptoses. All the PmrA LPSs, also that from the *rfaE* strain, bound ~50% less PM than the corresponding parent type LPSs (i.e., ~0.4 mol/mol LPS). This suggests that the *pmrA* mutation results in an altered form of the dOclA or lipid A region of LPS. Interestingly, the naturally occurring PM resistance in *Proteus* strains [31] is seen also in the heptoseless *Proteus mirabilis* R45 (M. V., unpublished). The LPS from this strain bound ~0.4 mol/mol LPS, a value comparable to that observed with LPS from the heptoseless *pmrA* *S. typhimurium* strain (0.38 mol/mol LPS, table 1).

Table 1
Polymyxin binding[a] to lipopolysaccharides from parent type and *pmrA* strains

Strain	Relevant genotype	PM bound mol/mol LPS[b]
SH 5014[c]	*rfaJ*	0.80
SH 6482[c]	*rfaJ*	0.86
SH 5357[c]	*rfaJ pmrA*	0.48
SH 6497[c]	*rfaJ pmrA*	0.43
SH 7334[c]	*rfaJ pmrA*	0.42
SH 7518[d]	*rfaE*	0.68
SH 7519[d]	*rfaE pmrA*	0.38
Proteus mirabilis R45	*rfaE*	0.41

[a] Determined as in section 2
[b] Molar amounts of LPS were quantitated on the basis of the glucosamine content (2 mol glucosamine = 1 mol LPS)
[c] Isogenic *S. typhimurium* derivatives described in [12] and in (M. V., submitted)
[d] Isogenic derivatives of *S. typhimurium SL* 1102 [15]

3.2. *SDS–Polyacrylamide gel electrophoresis of LPS*

The overall electrophoretic banding pattern of both the parent-type and PmrA LPS (fig.1) shows a basically similar heterogeneity in the number of O-specific repeating units [6,7]. The bands of both types of LPS occur as doublets, clearly seen in the lower part of the gel. Whereas in the parent type LPS most of the LPS (~90%) migrates as a faster band and the slower band of each doublet is scarcely visible, the slower band prevails (~80%) in the PmrA LPS. These results were found using exponential cells.

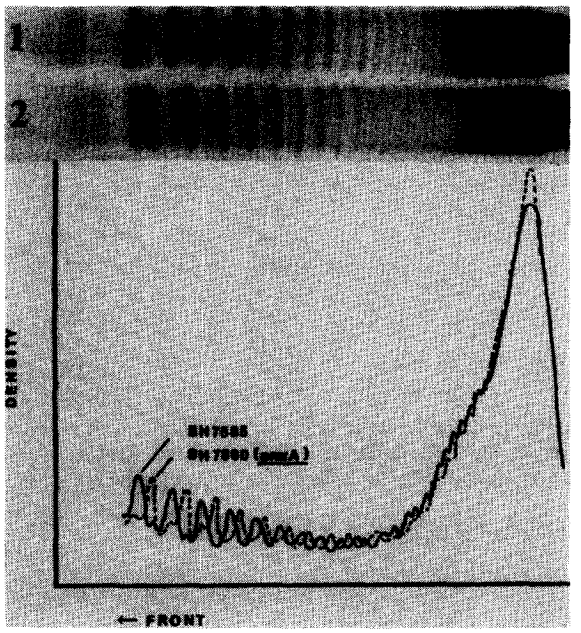

Fig.1. Electrophoretic analysis (autoradiogram and its densitometric scan) of the LPS from a parent-type strain (SH7585, slot 1) and a *pmrA* derivative (SH7580, slot 2). Bacterial cultures were grown in L broth ([32], without glucose) containing 1 mM fucose to optical density of 100 Klett units (Klett-Summerson colorimeter, red filter) and labeled with [^{14}C]-galactose (spec. act. 60 mCi/mmol) for 15 min, whereafter the envelope material was isolated by mild sonication and pelleted at 8000 × *g*. SDS–PAGE was done in 15% acrylamide as in [33]. The autoradiogram (Osray RP1 X-ray film, Agfa Gevaert) was scanned with a Helena densitometer (Helen Labs., TX).

Labeling of the cells in the stationary phase of growth gave predominant slower bands in both types of LPS (not shown). The double character of LPS bands has earlier been observed to be influenced by growth media [6] and storage [7] and interpreted to be probably caused by heterogeneity at the core-lipid A level. The electrophoresis results exclude large differences between the M_r of the PmrA and parent-type LPS, and thus also the possibility of heat-stable covalent linkages between the PmrA LPS molecules.

3.3. *Sugar and fatty acid analyses*

No differences were found in the fatty acid composition between the PmrA and parent-type LPS. The *pmrA* mutation did also not alter the glucose, galactose, heptose, phosphate or dOclA content of the RfaJ LPS (not shown).

Table 2
Content of ethanolamine containing compounds in parent-type of PmrA LPS

Strain	Ethanolamine containing compounds[a] (mol/mol LPS) released by	
	Mild acid[b]	Strong acid[c]
SH 5014 (*rfaJ*)	1.0	1.2
SH 6482 (*rfaJ*)	1.0	1.1
SH 5357 (*rfaJ pmrA*)	1.3	1.6
SH 6497 (*rfaJ pmrA*)	1.5	2.0

[a] As analyzed by the amino acid analyzer
[b] PPEtn and PEtn found after mild acid hydrolysis (0.2 N HCl, 15 min, 100°C)
[c] PEtn and Etn found after strong acid hydrolysis (4 N HCl, 10 h, 100°C)

3.4. *Phosphate substituents*

The PmrA LPSs comprised more ethanolamine-containing compounds (PEtn, PPEtn) than the parent-type LPSs (table 2). Part of these compounds was released from the LPS by mild acid treatment and thus were probably derived from pyrophosphoryl-ethanolamine linked to the reducing glucosamine residue of lipid A and/or to heptose (I) [10]. Additional PEtn and Etn was released after strong acid hydrolysis (4 M HCl) probably deriving from PPEtn, PEtn and dOclA-linked PEtn (table 2). Thus, the increase in ethanolamine containing compounds in the PmrA LPS appears to reflect an increased degree of substitution of at least two different phosphate groups of LPS.

The 4-ARAN content of the LPS (table 3) was analyzed by taking advantage of the fact that it, like 2-deoxysugars and also 4-amino-4,6-dideoxygalactose-TNP [34], reacts with diphenylamine [27]. *Proteus mirabilis* R45 LPS, containing 1 mol 4-ARAN/1 mol LPS (linked to the ester-bound lipid A phosphate; Z. Sidorczyk, M. J., E. Th. R., in preparation), was used as a reference. Mild acid hydrolysis of PmrA LPS released 0.56–0.67 mol 4-ARAN/mol LPS, which was 4–6-times more than the amount released from the corresponding parent type LPS preparations 0.1–0.15 mol/mol LPS). Slightly modified hydrolysis conditions (0.1 N or 0.02 N HCl, 10–60 min gave similar results.

The alkali-lability of the ester-bound phosphate group of the lipid A component of PmrA LPS (last 2 columns of table 3) suggested that the 4-ARAN was

Table 3
The content of 4-ARAN, and the alkali-lability of the ester-linked lipid A phosphate, in parent-type and *PmrA* LPS

Strain	GlcN (nmol/mg LPS)	4-ARAN[a,b] (mol/mol LPS)	4-ARAN[c] (mol/mol LPS)	Total phosphate in	
				Free lipid A (mol/mol)	free lipid A-OH[d] (mol/mol)
SH 5014 (*rfaJ*)	536	0.11	0.05	0.88[e]	0.71[e]
SH 6482 (*rfaJ*)	554	0.11	0.05	0.98[e]	0.74[e]
SH 7518 (*rfaE*)	758	0.15	–[f]	0.89	0.62
SH 5357 (*rfaJ pmrA*)	550	0.56	0.15	0.89[e]	0.31[e]
SH 6497 (*rfaJ pmrA*)	570	0.63	0.20	0.91[e]	0.25[e]
SH 7334 (*rfaJ pmrA*)	522	0.67	–	0.91[e]	0.21[e]
SH 7519 (*rfaE pmrA*)	798	0.58	–	0.93	0.23
P. mirabilis (*rfaE*)	798	1.00	–	0.92[e]	0.14[e]

[a] Molar amounts of LPS or lipid A were quantitated on the basis of the glucosamine content (2 mol GlcN = 1 mol lipid A)
[b] Calculated assuming the 4-ARAN content of *P. mirabilis* R45 to be 1 mol/mol LPS. Mean values from 2–3 experiments
[c] Analyzed by amino acid analyzer
[d] Released after sequential alkaline (0.17 N NaOH, 100°C, 1 h) and acid (0.17 N HCl, 100°C, 1 h) hydrolysis
[e] Analyses of 2–3 separate batches
[f] –, not determined

linked to this group (in analogy to *P. mirabilis*, *Chromabacterium violaceum* and *Rhodospirillum tenue*, see [10,35,36]). Therefore, LPSs from *pmrA* mutants and *P. mirabilis* were treated with alkali and the supernatant subjected to thin-layer chromatography (TLC). TLC revealed in both preparations one spot of comparable intensity with R_F values of 0.09 (solvent A) and 0.10 (solvent B). This spot was minute in TLC of alkali-treated parent type LPS. It contained phosphate, and stained yellow with ninhydrin. After treatment with 10% acetic acid and heating (120°C), the spot colored brown and gave a bright fluorescence at 366 nm. It is concluded that the material corresponding to the spot represents 4-ARAN phosphate.

The 4-ARAN determination by an amino acid analyzer gave similar difference between the PmrA and parent-type LPS, but the absolute amounts were ~3-times smaller. This could result from the lability of 4-ARAN [27] or from the occurrence of 4-ARAN in 2 forms, only one being found in the analysis, as suggested [27].

4. Discussion

This paper shows that LPS from PM resistant (*pmrA*) strains of *S. typhimurium* binds 2-times less PM than do LPSs from similarly grown parent-type strains and contains 4–6-times more 4-ARAN (0.6–0.7 mol/mol LPS) as a substituent of the ester-linked lipid A phosphate, as compared to the parent type LPS. This substitution of an acidic phosphate group could well account for the decreased binding of PM, a basic molecule. In analogy, the high 4-ARAN substitution in LPS could account for the natural polymyxin resistance of *Proteus*, already attributed to its outer membrane [34]. The PmrA LPS also comprised more ethanolamine-containing compounds than the parent type LPS.

The increased amount of 4-ARAN could explain the preponderance of the slower band of the doublets found in the electrophoresis of the PmrA LPS. It remains to be elucidated to what extent the other phosphate substituents contribute to the formation of this band.

In *Salmonella*, 4-ARAN substitutes usually 10–25% of the ester-bound lipid A phosphate [10,11,37,38]. This was also the case here. For *S. minnesota rfaE* strain, grown on mineral medium to stationary phase of growth, this figure was as high as 60% [10]. The 4-ARAN content (in lipid A precursor) is partially dependent on the growth medium [37]. It is possible that bacteria, depending on their physiological demands, are able to add (or omit) ionic headgroups, including 4-ARAN, and thus regulate their surface charge [10]. The *pmrA* mutation might represent the lack or an altered form of this regulation, leading to a constantly less anionic form of LPS.

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