Phosphate groups in lipopolysaccharides of *Salmonella typhimurium rfaP* mutants

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Abstract Lipopolysaccharides (LPS) of *Salmonella typhimurium rfaP* mutants and of a *galE* strain as a control were subjected to analysis by ^{31}P-NMR in order to assess the location of phosphate groups. This was done to obtain direct proof for our earlier finding by chemical analysis that phosphate was lacking in the core oligosaccharide part of the mutant LPS, whereas the core oligosaccharide normally contains several phosphate groups. Such phosphate deficiency has been associated with the increased susceptibility of the *rfaP* mutants to hydrophobic antibiotics and detergents. Analysis of the de-*O*-acylated LPS derivatives of *S. typhimurium rfaP* strains SH7770, SH8551, and SH8572 by ^{31}P-NMR revealed an almost total lack of phosphate groups in the core oligosaccharide part, the LPS phosphates being largely accounted for by the two monophosphate monoesters of lipid A, linked to positions C-1 and C-4′ of the lipid A backbone. Core oligosaccharide-linked phosphates were detected in minor proportions only, indicating the presence of some normally phosphorylated core oligosaccharide, due to the inherently leaky nature of the mutation.

Key words: *Salmonella typhimurium*; Structure; Lipopolysaccharide; Outer membrane; ^{31}P-NMR

1. Introduction

The outer membrane (OM) of Gram-negative bacteria functions as an effective barrier against external solutes, such as hydrophobic antibiotics [1,2]. The barrier function of OM is largely attributed to the presence of lipopolysaccharide (LPS) molecules, which create a hydrophilic surface due to the polysaccharide chains exposed on the membrane surface. The LPS are composed of a lipid portion (lipid A), to which is covalently linked a core oligosaccharide, typically carrying in its lipid A–proximal region (inner core) negatively charged groups such as carboxyl and phosphate groups that are believed to contribute to the integrity of the OM (for review of LPS structure, see [3]). As shown in Fig. 1 for *Salmonella*, some of the phosphates are partially substituted by cationic groups. The distal sugar chain (*O*-antigenic polysaccharide) linked to the core oligosaccharide appears to play no role in the permeability barrier function of the OM, and this applies also for the outer core oligosaccharide. However, mutations affecting the biosynthesis of the inner core typically result in a functionally defective OM, manifested as increased susceptibility of such mutant bacteria towards hydrophobic antibiotics [4,5].

In 1989 we described *rfaP* mutants of *Salmonella typhimurium* [6], which were shown to be analogous to the core phosphate-deficient *rfaP* mutant of *S. minnesota* described earlier by Mühlradt et al. [7]. The *rfaP* mutants were shown to be highly sensitive to certain hydrophobic antibiotics and to detergents, indicating that the permeability barrier function of the OM was impaired in these strains. In addition to their genetic and physiological characterization, their LPS were abnormal in that they contained only half of the phosphate expected for wild-type *S. typhimurium* LPS of the same chemotype. On the basis of chemical analyses the phosphate deficiency was restricted to the core oligosaccharide, and the impaired membrane function was attributed to this defect. Accordingly, phosphate in the mutant LPS was suggested to reside almost exclusively in the lipid A part, but the exact arrangement of phosphate groups was not studied. Phosphorus nuclear magnetic resonance (^{31}P-NMR) spectroscopy provides a powerful tool for investigation of the often complex and heterogenous architecture of LPS-linked phosphate groups, and the technique has been successfully applied to quantitatively analyze the phosphates in *Salmonella* LPSs [8–10]. In this article we report the results of reinvestigation of the *S. typhimurium rfaP* mutant LPS by ^{31}P-NMR.

2. Materials and methods

2.1. Bacteria and cultivation

Details of *S. typhimurium* SH6749 (*galE*, [11]), and the *rfaP* strains SH7770 (line LT2), SH8551 (line TML), and the derivative SH8572 (LT2) were described previously [6]. These strains, including SH6749 in the absence of extraneous D-galactose, elaborate LPS with a saccharide backbone mainly of chemotype Rc (see Fig. 1), along with smaller proportions of complete core (Ra) and smooth LPS material due to the inherent leakiness of the mutations [6]. All strains were cultivated on Luria agar as described previously [12].

2.2. LPS preparations

To prepare LPS, cells were washed successively with 94% ethanol, acetone, and diethyl ether, and extracted by the phenol/chloroform/petroleum ether method [13]. De-*O*-acylation of LPS was achieved by treatment with anhydrous hydrazine (TCI, Japan) for 20 min at 37°C [14].

2.3. Phosphorus magnetic resonance spectroscopy (^{31}P-NMR)

Samples of de-*O*-acylated LPS (dry wt., 15 mg) were dissolved in 0.6 ml of D_2O, which contained 2% sodium deoxycholate and 5 mM EDTA, and triethylamine was added until the solutions turned clear. To lower pD, dilute DCl was added. Spectra were recorded with a Varian 500 Unity instrument (11.7 T) at 27°C over a spectral range of 7000 Hz, with ^{1}H broadband decoupling (WALTZ-16) during acquisition. The pulse flip angle was 90°, and the relaxation delay between scans (300) was 20 s. The spectra were referenced to external 85% phosphoric acid (0.00 ppm).

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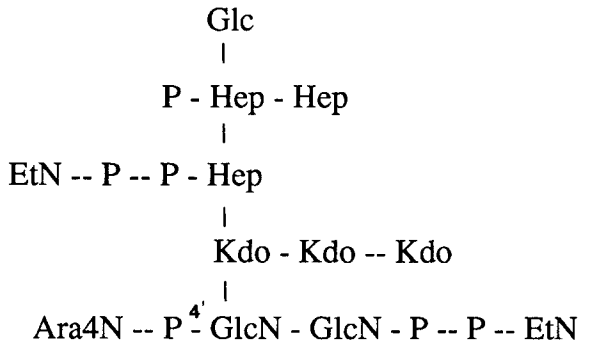

Fig. 1. Structure of the maximally phosphorylated and substituted saccharide backbone of *Salmonella* LPS of chemotype Rc [3]. The substituents of the primary phosphates are normally present in non-stoichiometric amounts only; dashed lines indicate partial substitution. The phosphorylated GlcN disaccharide constitutes the lipid A backbone, which carries four (*R*)-3-hydroxytetradecanoic acids (not shown) in ester and amide linkage, and dodecanoic and tetradecanoic acid linked to the 3-hydroxyl group of the (*R*)-3-hydroxytetradecanoic acids of the non-reducing GlcN. Ara4N, 4-amino-4-deoxy-L-arabinose; EtN, 2-aminoethanol; Glc, D-glucose; GlcN, 2-amino-2-deoxy-D-glucose; Hep, L-*glycero*-D-*manno*heptose; Kdo, 3-deoxy-D-*manno*octulosonic acid; P, phosphate.

3. Results and discussion

3.1. Structural and spectroscopic characteristics of normally phosphorylated Salmonella LPS

Fig. 1 shows the structure of the fully phosphorylated saccharide backbone of *Salmonella* LPS of chemotype Rc LPS. In LPS isolated from wild-type cultures, the primary phosphates carry further substituents (phosphate, 2-aminoethyl phosphate (EtN-P), 4-amino-4-deoxy-L-arabinose (L-Ara4N)) in non-stoichiometric proportions, giving rise to different phosphate arrangements at one position.

^{31}P-NMR spectra obtained from the de-*O*-acylated LPS of the *galE* strain SH6749 are shown in Fig. 2. These spectra were recorded at solvent pD values of 7.1 and 10.3, to assist identification of signals, since peaks corresponding for phosphate monoesters are characteristically shifted to higher ppm values at higher pD; it is also evident from Fig. 2 that discrete signals were obtained at pD 10.3, whereas in the spectrum recorded at pD 7.1 assignment was impossible due to overlapping peaks. The signals were assigned to distinct phosphate groups of LPS as described previously for *S. typhimurium* [10]. The de-*O*-acylated LPS of SH6749 yielded strong signals corresponding to monophosphate monoesters (P_m), observed at pD 10.3 in the region of 2–6 ppm. Of these signals, the one at 2 ppm was assigned to the glycosidically linked phosphate of lipid A (1-P_m) and the one at 4.5 ppm to the ester-linked phosphate monoester at position 4′ of lipid A (4′-P_m). The signals most downfield (> 5 ppm) originate from phosphate monoesters linked to the heptose region of the core oligosaccharide. Monophosphate diesters (P_d) yielded relatively small signals, the one at −1.5 ppm most likely representing the 4′-phosphate of lipid A which carries a L-Ara4N residue, and the one at 0.5 ppm remains unassigned, but it could be a core-linked phosphodiester. The finding of the phosphodiester with L-Ara4N was surprising, as this aminopentose was not de-

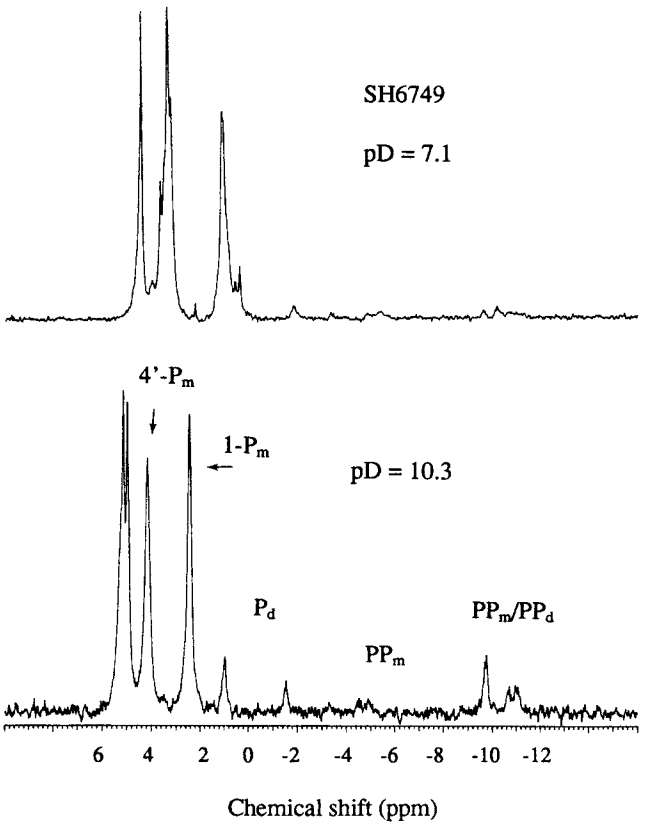

Fig. 2. ^{31}P-NMR spectra of de-*O*-acylated LPS of *S. typhimurium* SH6749 (*galE*) recorded at solvent (D_2O/sodium deoxycholate/EDTA) pD values of 7.1 and 10.3. P_m, monophosphate monoester; P_d, monophosphate diester; PP_m, diphosphate monoester; PP_d, diphosphate diester. Numbers refer to the positions of phosphate at the lipid A backbone (cf. Fig. 1).

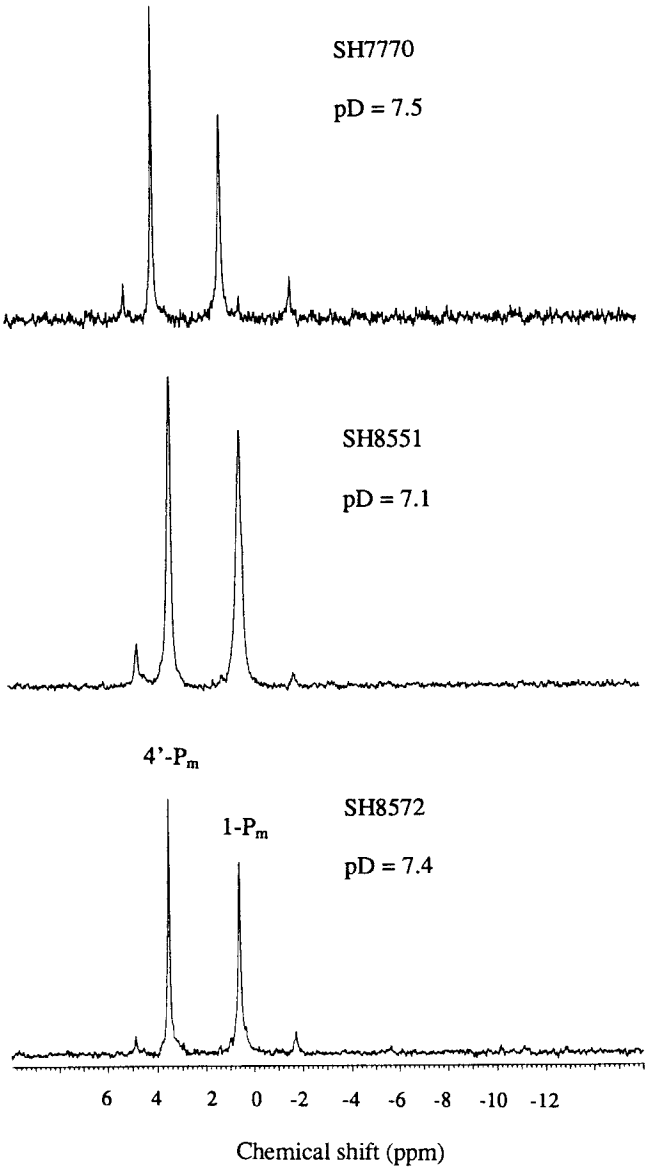

Fig. 3. ^{31}P-NMR spectra of de-*O*-acylated LPS of *S. typhimurium rfaP* strains SH7770, SH8551 and SH8572. Solvent pD value during the measurement is given for each spectrum; small differences in the chemical shifts are due to different pD values. Abbreviations and numbers as in Fig. 2.

tected by our earlier chemical analysis. This is obviously due to the difficulties in the detection of L-Ara4N due to its thermolability. In addition, signals corresponding to diphosphate monoesters and diesters (2-aminoethyl diphosphate) were present, these groups being linked to the core oligosaccharide and, to a small extent, to the glycosidic position of lipid A [10].

3.2. Analysis of the rfaP mutant LPS

The ^{31}P-NMR spectra of the de-*O*-acylated LPS of the *rfaP* strains (Fig. 3, recorded at neutral pD) were remarkably simple compared to that of SH6749. The spectra were dominated by the lipid A monophosphate monoester signals, the core-derived monophosphate signals being minor, and no diphosphates were present. Integration of the signals confirmed that the phosphates of the *rfaP* mutant LPS were almost completely accounted for by the lipid A-linked monophosphate monoester groups, accounting for 85%, 89%, and 93% of all phosphates of the LPS of SH7770, SH8551, and SH8572, respectively. For comparison, the lipid A-linked monophosphate monoesters amounted to only 46% of all phosphates of SH6749 LPS. The presence of small amounts of core-linked phosphate in *rfaP* LPS is in full agreement with the previously recognized fact that all *rfaP* mutants of *S. typhimurium* were leaky, producing some LPS with obviously normally phosphorylated complete core oligosaccharide (Ra-LPS) and some smooth material as well [6]. The inherent leakiness may result from a requirement of at least some normally phosphorylated core oligosaccharide for the integrity of the OM, LPS with a phosphate-deficient core alone probably being totally unable to create a functional OM. Phosphate groups in the core oligosaccharide are, however, not absolutely required for OM integrity. This is exemplified by certain bacterial species such as *Klebsiella pneumoniae*, whose LPS cores are devoid of phosphate [15–17]. Instead, these LPS cores contain uronic acid, which may fulfill the requirement

for a negative charge in this region in addition to those provided by Kdo, which is generally present in LPS.

The findings presented here for *S. typhimurium rfaP* mutants are consistent with those previously shown for the *E. coli rfaP* mutant [18] and thus give further credence for the view that the deep core structures as well as the genes involved in their synthesis are completely analogous. The antibiotic-supersusceptible phenotype of the *rfaP* mutants is well known and characterized, as discussed above. However, the *rfaP* mutants also have other interesting properties. The *E. coli* hemolysin HlyA that is normally secreted, by the signal peptide-independent process, is in an inactive conformation when secreted through the OM of the *rfaP* mutant [19]. Accordingly, the phosphates play an important role in determining in which conformation HlyA is secreted. This is clearly a finding that should be studied in more detail by using methods of structural biochemistry. Furthermore, the transfer of an F-like plasmid is much more ineffective when the recipient cell carries the *rfaP* mutation than when the recipient has other mutations that affect the core oligosaccharide part of the LPS. This suggests the presence of an adhesin at the F pilus tip that recognizes heptose-linked phosphate or 2-aminoethyldiphosphate [20].

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