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Structure of a highly phosphorylated lipopolysaccharide core in the Δ*algC* mutants derived from *Pseudomonas aeruginosa* wild-type strains PAO1 (serogroup O5) and PAC1R (serogroup O3)

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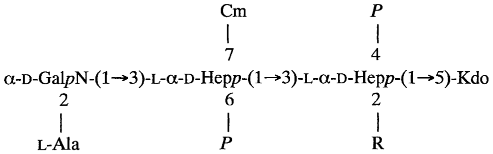
Abstract

Lipopolysaccharides (LPS) were isolated from rough-type mutant strains of *Pseudomonas aeruginosa* (Δ*algC*) derived from wild-type strains PAO1 (serogroup O5) and PAC1R (serogroup O3). Structural studies of the LPS core region with a special focus on the phosphorylation pattern were performed by 2D NMR spectroscopy, including a ^{1}H,^{31}P HMQC-TOCSY experiment, MALDI-TOF MS, and Fourier-transform ion cyclotron resonance ESIMS using the capillary skimmer dissociation technique. Both LPS were found to contain two residues each of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep), one residue of *N*-(L-alanyl)-D-galactosamine and one *O*-carbamoyl group (Cm) on the distal Hep residue. The following structures of a tetrasaccharide trisphosphate from strain PAC1R Δ*algC* and that carrying an additional ethanolamine phosphate group (*P*EtN) from strain PAO1 Δ*algC* were elucidated:



$$\begin{array}{lll}
 & \text{Cm} & P \\
 & | & | \\
 & 7 & 4 \\
\alpha\text{-D-Gal}p\text{N-}(1\to3)\text{-} & \text{L-}\alpha\text{-D-Hep}p\text{-}(1\to3)\text{-} & \text{L-}\alpha\text{-D-Hep}p\text{-}(1\to5)\text{-Kdo} \\
2 & 6 & 2 \\
| & | & | \\
\text{L-Ala} & P & \text{R}
\end{array}$$

where R = *P* in PAC1R Δ*algC* and *PP*EtN in PAO1 Δ*algC*.

To our knowledge, in this work the presence of ethanolamine diphosphate is unambiguously confirmed and its position established for the first time in the LPS core of a rough-type strain of *P. aeruginosa*. In addition, the structure of the complete LPS core of wild-type strain *P. aeruginosa* PAO1 was reinvestigated and the position of the phosphorylation sites was revised.

Keywords: Lipopolysaccharide; *Pseudomonas aeruginosa*; Core structures; Phosphorylation pattern; Ethanolamine diphosphate

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1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, which causes infections in immunocompromised patients and chronic pneumonia in patients with cystic fibrosis.[1,2] The extracellular alginate, rhamnolipid, and lipopolysaccharide (LPS) are important virulence factors of this bacterium.[3–5] In *P. aeruginosa,* several polysaccharides are associated with the LPS. The so-called A-band polysaccharide is a linear D-rhamnan.[6]

Abbreviations: Cm, carbamoyl; CSD, capillary skimmer dissociation; Hep, L-*glycero*-D-*manno*-heptose; HPAEC, high-performance anion-exchange chromatography; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; EtN, ethanolamine.

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The B-band polysaccharide (O-antigen) is a heteropolymer with repeating units composed of one to five glycosyl residues, whose structure varies from strain to strain and determines the bacterial serogroup and subgroup.[7] The LPS core of *P. aeruginosa* is a higher oligosaccharide, which includes a highly phosphorylated inner region consisting of residues of 3-deoxy-D-*manno*-octulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep) and an outer region containing D-glucose, L-rhamnose and D-galactosamine as well as *N*-(L-alanyl) and *O*-carbamoyl (Cm) groups.[8,9] The carbohydrate moiety in the R-type LPS is represented by a complete or incomplete core, and in the SR- and S-type LPS the core is substituted with one or several O-antigen repeating units, respectively.

The Δ*algC* gene product is common in the biosynthesis pathways of the LPS,[10–12] alginate,[12,13] and rhamnolipid.[5] This gene encodes two enzymes, phosphomannomutase, which interconverts mannose-6-phosphate and mannose-1-phosphate,[12] and phosphoglucomutase, which interconverts glucose-6-phosphate and glucose-1-phosphate.[10,14] The last compound is presumably an essential intermediate that is required for the addition of glucose residues to the LPS outer core region to complete biosynthesis of the core and to enable the attachment of the O-antigen.[10,11,15,16] Genetically defined Δ*algC* mutants have been constructed from *P. aeruginosa* wild-type strains PAO1 (serogroup O5) and PAC1R (serogroup O3) and shown by SDS-PAGE to express neither B-band nor A-band polysaccharides.[10] As opposite to the parent strain, the *P. aeruginosa* PAO1 Δ*algC* mutant was found to be avirulent in a burned-mouse model of infection. This was specifically assigned to the defect in the LPS biosynthesis since an alginate-specific mutation did not affect virulence.[17]

The LPS core has been chemically studied in a number of rough- and smooth-type strains of *P. aeruginosa*[18–25] with the aim to delineate the events that are involved in the LPS biosynthesis and to correlate the core structure with the immunospecificity and biological functions, particularly, as a ligand that is recognised by receptors of the lung epithelial cells.[26] It has been suggested[10] that the LPS structure of the Δ*algC* mutants differs from the structure of the wild-type strains in a similar manner as that of *P. aeruginosa* AK1012, which is a phosphomannomutase-deficient rough-type mutant derived from *P. aeruginosa* PAO1 after selection for resistance to LPS-specific bacteriophages.[27] However, none of the Δ*algC* mutants has been analysed in respect to the core structure in detail. In order to elucidate the influence of the genetically defined Δ*algC* mutation on the LPS structure we analysed the core oligosaccharide in the Δ*algC* mutants derived from *P. aeruginosa* PAO1 and PAC1R as well as that in the PAO1 parent strain. A special focus was made on the phosphorylation pattern of the core since published data on the distribution of the phosphorylation sites are contradictory.[18–24]

2. Results and discussion

The LPS were isolated from *P. aeruginosa* wild-type strains PAO1 (serogroup O5) and PAC1R (serogroup O3) and the respective Δ*algC* mutants and investigated by SDS-PAGE. Both mutants lacked the characteristic O-antigen ladder-like banding pattern that was observed for both parent strains. A faster mobility of the low-molecular mass LPS species from the Δ*algC* mutants, which correspond to the core-lipid A moiety, is consistent with published data.[10,11]

Data of compositional analyses of the LPS from the four *P. aeruginosa* strains are compiled in Table 1. The LPS of the wild-type strains are characterised by a high content of glucose and rhamnose, which are components of the outer core region.[28,29] In addition, rhamnose is present in the A-band polysaccharide (D-isomer) of both wild-type strains[6] and in the B-band polysaccharide (L-isomer) of strain PAC1R.[30] In contrast, in the LPS of the Δ*algC* mutants glucose is absent and rhamnose is present in a negligible amount that is in agreement with the previously proposed LPS structure.[10,11] The B-band polysaccharides were isolated by GPC after mild acid

Table 1
Composition of *P. aeruginosa* LPS (nmol mg^{-1})

	P. aeruginosa strain			
	wild-type		Δ*algC* mutant	
	PAO1	PAC1R	PAO1	PAC1R
Kdo	443	457	251	258
Total HexN [a]	630	662	571	555
P	1780	1437	2390	2160
GalN [b]	127	129	299	250
GlcN [b]	261	343	596	505
Ala	166	188	308	270
(*P*)EtN	17	0	199	41
Hep	308	272	122	125
Glc	746	504	0	0
Rha	428	315	37	26
10:0(3-OH)	211	178	254	393
12:0	174	76	73	91
12:0(2-OH)	166	270	434	464
12:0(3-OH)	292	284	413	514
16:0	14	54	0	23

[a] Determined by the Morgan–Elson reaction.

[b] Determined by high performance liquid chromatography (HPLC).

degradation of the LPS from wild-type strains PAO1 and PAC1R. Analysis using ^{1}H and ^{13}C NMR spectroscopy confirmed that their structures are identical to the reported structures of the O-antigens of the respective subgroups of *P. aeruginosa* O5 and O3, respectively[7,30,31] (data not shown).

Mass spectra of the intact LPS from the Δ*algC* mutants were complex owing to the presence of multiple adduct ions and heterogeneity in respect to the number of fatty acids and phosphate substituents. Therefore, O-deacylated LPS were prepared by mild hydrazinolysis, which removed selectively ester-linked fatty acids, and purified by electrodialysis. The ESI mass spectra of the O-deacylated LPS from both mutants showed the lowest-mass molecular peak at *m*/*z* 2235.7 and a fragment ion peak at *m*/*z* 896.4, thus indicating common structural features of the LPS. Presumably, the *m*/*z* 896.4 ion was induced by in-source fragmentation and corresponds to the lipid A backbone (bisphosphorylated glucosamine disaccharide) with two amide-linked 3-hydroxydodecanoyl groups as reported earlier for *P. aeruginosa*.[32–34] The difference of 1339.3 Da between the masses of the two common ions could be attributed to a core oligosaccharide composed of two Kdo residues, one residue each of Hep, HepCm and GalNAla, and three phosphate groups (calculated average molecular mass 1357.3 Da). This finding corresponds to published data of the inner core structure of the *P. aeruginosa* LPS.[18,20,21,24]

In addition, the ESI mass spectra showed multiple peaks in the region up to 2500 Da for higher-phosphorylated compounds and the corresponding sodium (+22 Da) and potassium (+38 Da) adduct ions. The observed mass differences of 80 and 123 Da correspond to the presence of an additional phosphate or ethanolamine phosphate (*P*EtN) or both groups. The highest-mass molecular ion peak in the spectrum of the O-deacylated LPS from PAO1 Δ*algC* mutant appeared at *m*/*z* 2438.6 and belonged to a heptakisphosphorylated LPS species that contains one ethanolamine group. In contrast, no or only a negligible amount of ethanolamine-containing species was detected in the O-deacylated LPS from PAC1R Δ*algC* mutant, and the highest-mass molecular ion peak for a heptakisphosphorylated LPS species was observed at *m*/*z* 2395.6. Considering that lipid A is bisphosphorylated, up to five phosphate groups occur in the LPS core region of both mutant strains.

The ^{31}P NMR spectra of the O-deacylated LPS from both Δ*algC* mutants run for solutions in D_2O showed intense signals near -10 ppm for diphosphate groups. These samples were not suitable for detailed analysis by ^{1}H and ^{13}C NMR spectroscopy owing to a low solubility of O-deacylated LPS in D_2O and line broadening. Therefore, the LPS were subjected to mild acid degradation, and the core oligosaccharides thus released were isolated by GPC on Sephadex G-50 and characterised by MALDI-TOFMS (Fig. 1). The mass spectra demonstrated highly phosphorylated core tetrasaccharides lacking one of the Kdo residues, which occupied the lateral position in the initial LPS and was cleaved during mild acid hydrolysis. In the core from PAO1 Δ*algC* mutant (Fig. 1, left panel), the major peaks were observed for tetrasaccharides with two to four phosphate groups and an additional ethanolamine group, whereas minor peaks were for compounds with five phosphate groups. In the core from PAC1R Δ*algC* mutant (Fig. 1, right panel), the major peaks belonged to tetrasaccharides with two to four phosphate groups but only minor peaks were present for compounds with an additional ethanolamine group.

The core oligosaccharides from wild-type strain PAO1 and both Δ*algC* mutants were fractionated by GPC on TSK HW-40 followed by high-performance anion-exchange chromatography (HPAEC) on CarboPac PA1 under neutral conditions. Despite a complexity of the chromatograms (Fig. 2), which could be expected from the MS data, several groups of fractions could be identified based on the repeated peak patterns, which were pooled and desalted by GPC on Sephadex G-50. MALDI-TOFMS (Fig. 2) revealed clusters of peaks that correspond to the core tetrasaccharides with different degrees of phosphorylation (up to four or three phosphate groups in the core from PAO1 Δ*algC* and PAC1R Δ*algC* mutants, respectively). The tetrasaccharides from PAO1 Δ*algC* mutant differ also in the presence or absence of EtN. No higher-phosphorylated core tetrasaccharides, which could be expected based on the MS data of the O-deacylated LPS and non-fractionated core oligosaccharides (see above), were isolated.

The isolated core oligosaccharide from PAO1 Δ*algC* mutant with the highest phosphate content was analysed by ESIMS in the negative ion mode. The mass spectrum revealed the major cluster with a peak at *m*/*z* 1260.21, which corresponds to a tetrasaccharide composed of one residue each of Kdo, Hep, HepCm and GalNAla, one ethanolamine and four phosphate groups (calculated average molecular mass 1260.21 Da). Peaks at *m*/*z* 1282.19 and 1298.16 in the cluster were attributed to the corresponding sodium and potassium adduct ions, respectively. The capillary skimmer dissociation (CSD) mass spectrum showed various kinds of fragment ions. Peaks at *m*/*z* 1179.24 and 1099.28 resulted from a stepwise loss of two phosphate groups, and those at *m*/*z* 1056.23 and 976.27 from a further loss of a *P*EtN group. Ions peaks at *m*/*z* 564.15 ($C2_{1P}$) arising from the C2 cleavage (Fig. 3) and that at *m*/*z* 521.14 from a further loss of Cm clearly indicated the presence of at least one phosphate group on HepCm. In addition, there were fragment ion peaks at *m*/*z* 695.19 ($B3_{1P}$) and 836.18 ($C3_{2P}$) arising from the B3 and C3 cleavages, respectively. No phosphorylated Y or Z ions from the

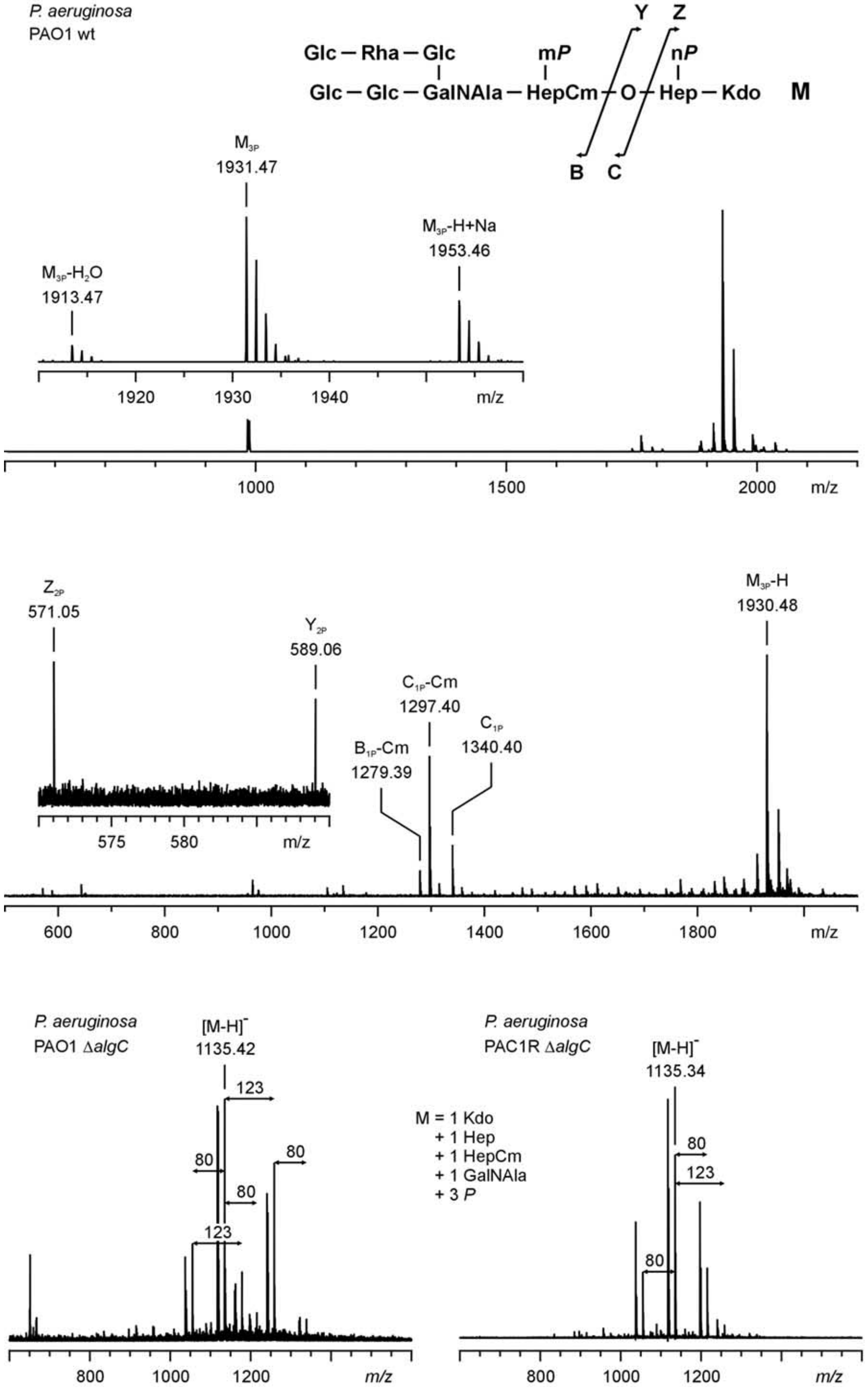

Fig. 1. MALDI-TOF mass spectra obtained in the negative ion mode of the core oligosaccharide mixtures obtained by mild acid hydrolysis of the LPS from *P. aeruginosa* PAO1 Δ*algC* and PAC1R Δ*algC*. Mass differences of 80 and 123 Da refer to phosphate and *P*EtN, respectively; other, not annotated peaks, most likely, are derived from anhydro–lactone forms (− 18 Da).

reducing end of the core were observed, which could be accounted for by a loss of phosphate groups upon fragmentation.

NMR spectroscopy studies of the core tetrasaccharide, including ^{1}H,^{1}H COSY, TOCSY, NOESY, ^{1}H,^{13}C HMQC and ^{1}H,^{31}P HMQC (Fig. 4) experiments, revealed a good correlation of the ^{1}H, ^{13}C and ^{31}P NMR chemical shifts (Table 2) and connectivities in the 2D NMR spectra with published data of *P. aeruginosa* strains studied earlier.[18–21,24] Particularly, the spectra showed the presence of Cm, Ala and EtN, and the position of Cm at O7 of Hep^{II} was specially confirmed

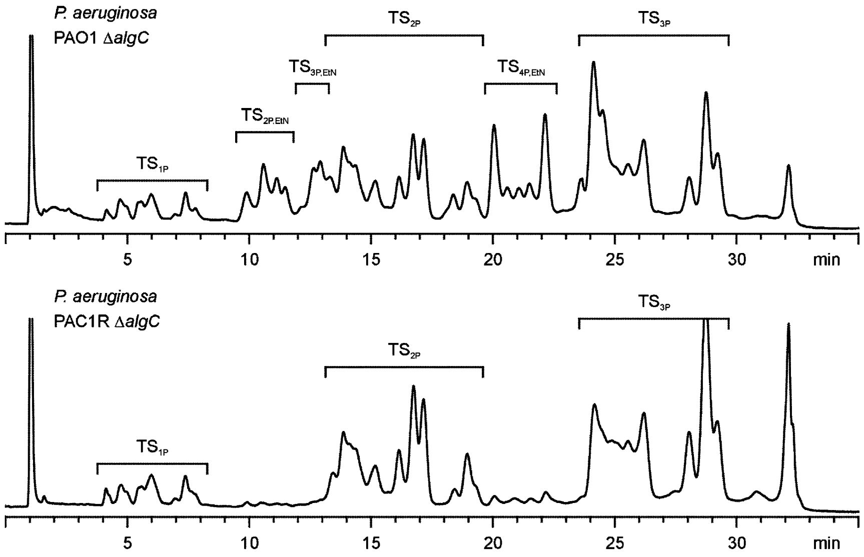

Fig. 2. Analytical HPAEC of the core oligosaccharide mixtures obtained by mild acid hydrolysis of the LPS from *P. aeruginosa* PAO1 Δ*algC* and PAC1R Δ*algC*. Brackets indicate groups of fractions that correspond to the compounds having the same molecular mass as determined by MALDI-TOFMS. TS, tetrasaccharide.

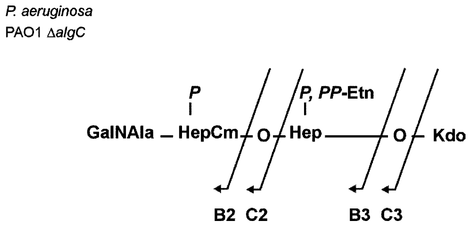

Fig. 3. Fragmentation pattern observed in the CSD mass spectrum of the *PP*EtN-containing core tetrasaccharide from *P. aeruginosa* PAO1 Δ*algC* isolated by HPAEC.

by $^{3}J_{C,H}$ coupling of H-7a,7b of Hep^{II} to the Cm carbon at δ_C 159.3 in the ^{1}H,^{13}C HMBC spectrum.

A ^{1}H,^{31}P HMQC-TOCSY experiment (Fig. 4) demonstrated the attachment of EtN to a diphosphate group at position 2 of Hep^{I}, i.e., defined the presence and the location of *PP*EtN. This followed from $^{3}J_{P,H}$ and $^{4}J_{P,H}$ couplings of P_α-2 at δ_P −9.48 to H-2 and H-1 of Hep^{I} at δ_H 4.74 and 5.26 as well as of P_β-2 at δ_P −9.75 to H-1 and H-2 of EtN at δ_H 4.15 and 3.24, respectively. Another phosphate group at δ_P 2.55 gave cross-peaks with H-4 of Hep^{I} in the ^{1}H,^{31}P HMQC spectrum and with H-2, H-3 and H-4 of Hep^{I} in the ^{1}H,^{31}P HMQC-TOCSY spectrum (Fig. 4), thus indicating unambiguously phosphorylation of Hep^{I} at position 4 too. The third phosphate group is bound to Hep^{II} at position 6 as the ^{1}H,^{31}P HMQC experiment revealed $^{3}J_{P,H}$ and $^{4}J_{P,H}$ couplings of P-6 at δ_P 1.89 to H-6 (strong) and H-7b (week) of Hep^{II} at δ_H 4.46 and 3.79, respectively. This finding was confirmed by the ^{1}H,^{31}P HMQC-TOCSY spectrum, which showed additional correlations of P-6 to H-7a (strong), H-4 and H-5 (both week) of Hep^{II} at δ_H 4.46, 3.87 and 3.96, respectively (Fig. 4).

Based on the MS and NMR spectroscopy data, it can be concluded that the core tetrasaccharide from strain PAO1 Δ*algC* has the structure shown in Fig. 5. Similar studies of the core tetrasaccharide from PAC1R Δ*algC* mutant showed that it has the same structure except for that Hep^{I} carries at position 2 a monophosphate group rather than *PP*EtN (Fig. 5). Accordingly, no signals for diphosphate and ethanolamine were present in the NMR spectra of the core tetrasaccharide from PAC1R Δ*algC*. The ^{1}H, ^{13}C and ^{31}P NMR chemical shifts were similar to those from PAO1 Δ*algC*, except for that of P-2 of Hep^{I}, which resonated at δ_P 1.92.

Although in past, detection of *P*EtN in LPS hydrolysates gave cause for speculations about the presence of a (*P*)*P*EtN group(s) in the *P. aeruginosa* core, this was confirmed for the first time in the present work and the position of *PP*EtN unambiguously established in *P. aeruginosa* PAO1 Δ*algC* mutant. The single *PP*EtN group was found to be linked to Hep^{I} at position 2 which had been previously reported as one of the phosphorylation sites in the LPS core of *P. aeruginosa*.[19–24] The same position of *PP*EtN has been recently demonstrated in the core of an S-type LPS from *P. aeruginosa* immunotype 5 (serogroup O10) (Bystrova, O.V. et al. *Biochemistry (Moscow)* in press) and seems to be a conserved feature of *P. aeruginosa*. A failure of earlier attempts to establish the location of diphosphate and *PP*EtN groups in the LPS of *P. aeruginosa* was attributed to their cleavage 'even under extremely mild conditions'.[23] In part this was confirmed in the present work as no core oligosaccharide with diphosphate monoester (-*PP*) could be isolated, most

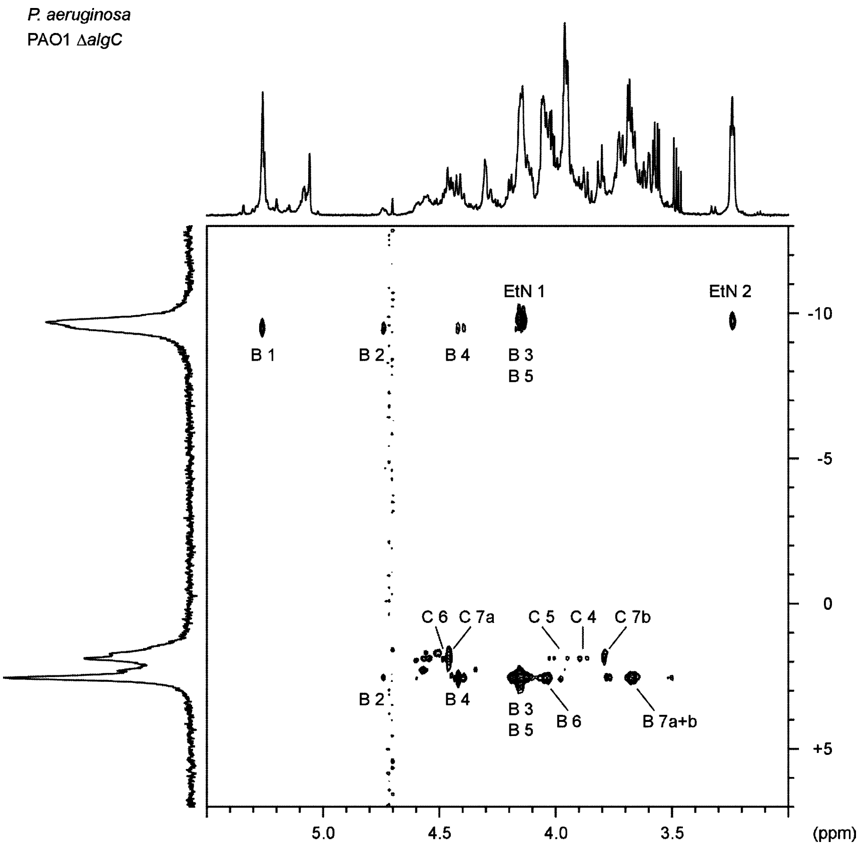

Fig. 4. Part of a ^{1}H,^{31}P HMQC-TOCSY spectrum of the *PP*EtN-containing core tetrasaccharide from *P. aeruginosa* PAO1 Δ*algC* isolated by HPAEC. The ^{31}P NMR spectrum and the corresponding part of the ^{1}H NMR spectrum are displayed along *F*1 and *F*2 axes, respectively. Numerals refer to atoms in sugar residues denoted by letters as shown in Fig. 5.

likely, owing to its high lability. In contrast, diphosphate diester (-*PP*EtN) largely survived both mild hydrazinolysis and mild acid hydrolysis of the LPS (Sánchez Carballo, P.M.; Zähringer, U. unpublished results).

Previously, *P*EtN and *PP*EtN have been found in the heptose-containing inner core region of several *Enterobacteriaceae*, including *Salmonella enterica*, *Escherichia coli*, *Shigella*, *Citrobacter*, and *Hafnia.* In most cases, this is *PP*EtN linked to Hep$^{\mathrm{I}}$ at position 4.[8] Two *P*EtN groups have been identified in the inner core region of the *Neisseria meningitidis* LPS and shown to be associated with a phase-variable epitope of a *P*EtN-specific monoclonal antibody.[35] Binding of one of the cross-reactive polyclonal antibodies against the inner core region of the *N. meningitidis* LPS has been demonstrated to be dependent on *P*EtN too.[36] As the other non-sugar substituents in the core, like *O*-carbamoyl and *N*-(L-alanyl) groups,[37] *PP*EtN in the *P. aeruginosa* LPS is a potential target for cross-protective monoclonal antibodies that are specific to the conserved structural elements of the LPS.

The distribution of the phosphorylation sites between Hep$^{\mathrm{II}}$ and Hep$^{\mathrm{I}}$ in the LPS core of Δ*algC* mutants of *P. aeruginosa* PAC1R and PAO1 is the same as reported earlier for R- and S-type LPS of certain *P. aeruginosa* strains[18,21,24] but differs from that of several other *P. aeruginosa* strains, including wild-type strain PAO1, in which the three phosphorylation sites have been attributed to Hep$^{\mathrm{I}}$.[19,20,22] In order to clarify whether this discrepancy is due to a strain difference or resulted from a misinterpretation of structural data, we reinvestigated the structure of the LPS core of *P. aeruginosa* PAO1.

The negative ion mode ESI mass spectrum of a core oligosaccharide isolated by HPAEC from the mild acid-degraded LPS of wild-type strain *P. aeruginosa* PAO1 showed the major molecular ion peak at *m*/*z* 1931.47. This peak has been attributed previously to a nonasaccharide composed of one residue each of Kdo, Hep, HepCm, GalNAla and Rha, four Glc residues and three phosphate groups.[20] The CSD mass spectrum (Fig. 6) showed a peak at *m*/*z* 1340.40 (C_{1P}) arising from the C cleavage, as well as peaks at *m*/*z* 1297.40 and 1279.39, which were due to a loss of Cm from the C and B fragments, respectively. All these fragments contain one phosphate group. The Z and Y cleavages resulted in ions at *m*/*z* 571.05 (Z_{2P}) and 589.06 (Y_{2P}), indicating that the Hep$^{\mathrm{I}}$–Kdo disaccharide fragment from the reducing end of the core contained two phosphate groups. No Y or Z fragment ions with three phosphate groups at Hep$^{\mathrm{I}}$ were detected.

Table 2
^{1}H, ^{13}C, and ^{31}P NMR chemical shifts (δ, ppm) of the core tetrasaccharide and nonasaccharide obtained by mild acid hydrolysis of the LPS from *P. aeruginosa* PAO1 Δ*algC* mutant and PAO1 wild-type strain followed by HPAEC

Residue	Nucleus	1	2	3		4	5	6		7		8	
				3ax	3eq			6a	6b	7a	7b	8a	8b
Tetrasaccharide													
Kdo	^{1}H			2.17	1.84	4.06	4.06	3.69		3.81		3.57	3.48
	^{13}C		99.5	34.3		66.9	73.8	73.2		71.4		63.7	
Hep^{I}	^{1}H	5.26	4.74	4.16		4.42	4.15	4.03		3.68	3.68		
	^{13}C	99.9	76.0	75.9		71.3	73.0	69.7		63.6			
	^{31}P		α -9.48 β -9.75			2.55							
EtN	^{1}H	4.15	3.24										
	^{13}C	63.5	41.4										
Hep^{II}	^{1}H	5.06	4.30	3.97		3.87	3.96	4.46		4.46	3.79		
	^{13}C	103.8	70.6	79.2		67.1	71.6	70.5		62.2			
	^{31}P							1.89					
Cm	^{13}C	159.3											
GalN	^{1}H	5.26	4.12	3.95		3.96	4.05	3.72	3.68				
	^{13}C	99.9	51.6	68.4		69.7	72.4	62.1					
Ala	^{1}H		4.01	1.47									
	^{13}C	172.5	50.5	17.7									
Nonasaccharide													
Hep^{I}	^{1}H	5.15	4.64	4.14		4.44	4.13	4.03		3.67	3.67		
	^{13}C	99.8	74.9	75.7		70.7	72.9	69.5		63.4			
	^{31}P		1.91			2.95							
Hep^{II}	^{1}H	5.06	4.33	3.97		3.96	3.86	4.46		4.43	3.82		
	^{13}C	103.8	70.3	78.7		66.7	70.9	71.2		62.2			
	^{31}P							2.57					
Cm	^{13}C	162.3											

The ^{1}H, ^{13}C and ^{31}P NMR chemical shifts of the core oligosacharide from *P. aeruginosa* PAO1 were similar to the published data[19,20] (Table 2). Moreover, the sugar linkages and sequence (Fig. 5), which were determined by 2D NMR $^{1}H,^{1}H$ and $^{1}H,^{13}C$ shift-correlation experiments, were found to be identical. A $^{1}H,^{31}P$ HMQC experiment showed Hep^{I} P-2/H-2, Hep^{I} P-4/H-4 and Hep^{II} P-6/H-6 cross-peaks at δ_P/δ_H 1.91/4.64, 2.95/4.44 and 2.57/4.46, respectively, due to $^{3}J_{P,H}$ coupling and a Hep^{II} P-6/H-7a cross-peak at δ_P/δ_H 2.57/4.43 due to $^{4}J_{P,H}$ coupling. The phosphorylation pattern was confirmed by the $^{1}H,^{31}P$ HMQC-TOCSY spectrum (Fig. 7), which showed correlations for Hep^{I} P-2 and P-4 with Hep^{I} H-2, H-3 and H-4 and for Hep^{II} P-6 with Hep^{II} H-7a and H-7b.

The data obtained indicate the same phosphorylation pattern of the LPS core in both wild-type PAO1 and PAO1 Δ*algC* mutant strains with two phosphorylation sites at Hep^{I} and one site at Hep^{II}, and this pattern seems to be a conserved feature of *P. aeruginosa*. Most likely, the previous erroneous allocation of one of the phosphate groups[19,20] resulted from misassignment of the signals for H-6 and H-7 of Hep^{I} and Hep^{II} in the ^{1}H NMR spectrum of the core oligosaccharide.

Remarkably, the core oligosaccharide of the parental PAO1 strain contains significantly less *P*EtN as compared with its Δ*algC* mutant. The degree of substitution with EtN is significantly different also in PAC1R and the corresponding Δ*algC* mutant. The biochemical significance of this observation remains so far unclear.

3. Experimental

3.1. Bacterial strains and growth

The Δ*algC* mutants derived from *P. aeruginosa* wild-type strains PAO1 from serogroup O5[27,29] and PAC1R from serogroup O3[28,38] were described by Coyne and co-workers.[10] All strains were grown for 12–18 h under agitation (140 rpm) at 37 °C in Luria–Bertani medium consisting of aq 1% (w/v) tryptone, 1% (w/v) sodium chloride, and 0.5% (w/v) yeast extract, adjusted to pH 7.0. Cells were killed by addition of aq 1% (w/v) phenol,

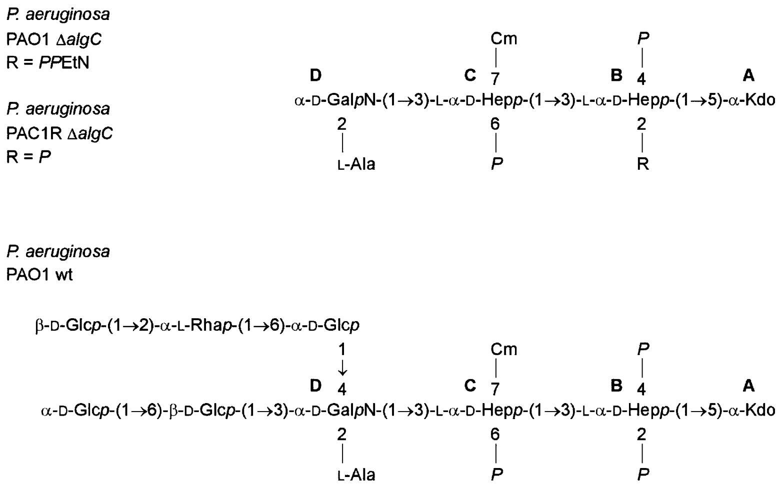

Fig. 5. Structures of the major core tetrasaccharides from *P. aeruginosa* PAO1 Δ*algC* and PAC1R Δ*algC* mutants and the core nonasaccharide from *P. aeruginosa* wild-type strain PAO1.

centrifuged, washed two times each with EtOH, acetone, and ether, and left for drying at rt.

3.2. Extraction of LPS

Dried cells were suspended in 5 mM Tris–HCl buffer pH 7.5 containing 2 mM calcium chloride, treated with DNAse A and RNAse A at 37 °C for 24 h and then with proteinase K (all enzymes from Boehringer Mannheim) under the same conditions for another 24 h. The suspensions were dialysed using a 12–16-kD MWCO membrane (Biomol) against distilled water at 4 °C for 5 days and lyophilised.

LPS of wild-type strains were extracted by the hot phenol–water procedure[39] in yields 1.6 and 0.9 g LPS from 22.0 and 18.0 g enzyme-treated dried cells of strains PAO1 and PAC1R (7.2 and 5.2%, respectively). LPS of mutant strains were extracted by the phenol–$CHCl_3$–petroleum ether procedure[40] in yields of 1.5 and 1.7 g LPS from 20.7 and 32.4 g enzyme-treated dried cells of strains PAO1 Δ*algC* and PAC1R Δ*algC* (7.3 and 5.2%, respectively).

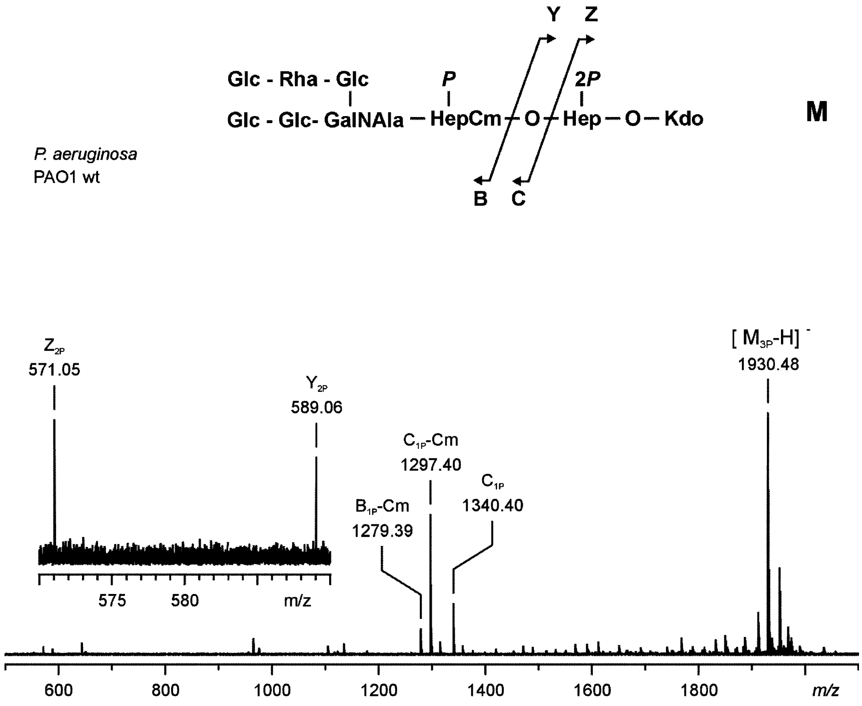

Fig. 6. CSD mass spectrum of the core nonasaccharide trisphosphate from *P. aeruginosa* wild-type strain PAO1 isolated by HPAEC. The inset on the CSD mass spectrum shows an enlargement of the region for the Y and Z fragment ions. The fragmentation pattern explaining the origin of fragment ions observed in the CSD mass spectrum is shown in the top inset.

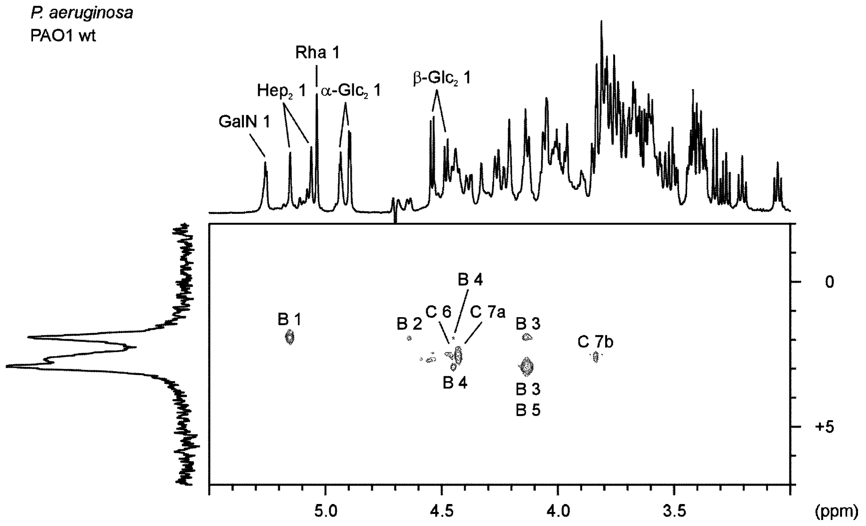

Fig. 7. Part of a ^{1}H,^{31}P HMQC-TOCSY spectrum of the core nonasaccharide from *P. aeruginosa* wild-type strain PAO1 isolated by HPAEC. The ^{31}P NMR spectrum and the corresponding part of the ^{1}H NMR spectrum are displayed along *F*1 and *F*2 axes, respectively. Numerals refer to atoms in sugar residues denoted by letters as shown in Fig. 5.

3.3. SDS-PAGE

SDS-PAGE was performed using 4% (w/v) polyacrylamide stacking gel (8 × 2 cm) and 20% (w/v) polyacrylamide separating gel (8 × 5 cm) as described.[41] 1 µg LPS from each strain was applied to the gel, which was fixed after developing and silver-stained.[42]

3.4. Compositional analyses

The content of Kdo was determined by the modified thiobarbituric acid assay.[43] The total of hexosamines was determined after acid hydrolysis (4 M HCl, 100 °C, 16 h) using the Morgan–Elson reaction as described.[44] The total phosphate was determined by the ascorbic acid method.[45] GLC was performed with a Hewlett–Packard model 5890 Series II instrument equipped with a 30-m capillary column of SPB-5 (Supelco) using a temperature gradient of 150–320 °C at 5 °C min^{-1}. Neutral sugars were analysed by GLC as the alditol acetates after hydrolysis with 0.1 M HCl at 100 °C for 48 h.[46] Fatty acids were analysed by GLC as the methyl esters prepared by methanolysis of the LPS with 2 M HCl in methanol at 120 °C for 24 h. Amino acids, hexosamines, and (phospho)ethanolamine were analysed as the phenylthiocarbamoyl derivatives after hydrolysis of the LPS with 8 M HCl at 100 °C for 16 h and separation with a Waters HPLC instrument equipped with a 3.9 × 150 mm column of Pico Tag (Waters).

3.5. O-deacylation of LPS

LPS (100 mg from each Δ*algC* mutant) was treated with anhydrous hydrazine (5 mL) at 37 °C for 30 min. The samples were cooled with ice and 50 mL ice-cold acetone was added. After concentration under diminished pressure, the samples were electrodialysed using a 1-kD MWCO membrane (Amika) in a 1.5-mL microelectrodialysis chamber (Amika) against 20 mM ammonium bicarbonate buffer pH 9.0 at 150 V (~ 20 mA) and 4 °C for 2 h and lyophilised.

3.6. Mild acid degradations of LPS

LPS from each strain was dissolved in 0.1 M AcONa buffer pH 4.4 at a concentration 10 mg mL^{-1} and heated at 100 °C for 4 h. The precipitate was removed by centrifugation and the supernatant desalted by GPC on a column (2.5 × 50 cm) of Sephadex G-50 (Amersham Biosciences) using pyridinium acetate buffer pH 4.3 (4 mL pyridine and 10 mL AcOH in 1 L water) and a differential refractometer (Knauer) for detection. Fractionation by GPC on a column (2.5 × 120 cm) of Toyopearl TSK HW-40 (TosoHaas) using the same buffer and detector afforded high-molecular-mass polysaccharides (from wild-type strains only) and core oligosaccharides (from all strains).

3.7. High-performance anion-exchange chromatography

The major core oligosaccharide fractions from strains PAO1 (55.0 mg, three runs), PAO1 Δ*algC* (24.8 mg, two runs) and PAC1R Δ*algC* (38.4 mg, three runs) were fractionated by HPAEC on a semi-preparative column (9 × 250 mm) of CarboPac PA 1 (Dionex) using a linear gradient (2–52%) of 1 M AcONa pH 6.0 in water for 140 min at 1.5 mL min^{-1} and 12–18 mg material per run. Three millilitres fractions were collected and a 99 µL-aliquot of each fraction was analysed by HPAEC on an analytical column (4.6 × 250 mm) of CarboPac PA 1 (Dionex) using a linear gradient (2–50%) of 1 M AcONa pH 6.0 in water for 30 min at 1.5 mL min^{-1} and a pulsed amperometric detector (Dionex). Fractions were appropriately pooled and desalted by GPC on Sephadex G-50 as described above.

3.8. Mass spectrometry

MALDI-TOFMS of core oligosaccharides before and after fractionation by HPAEC was performed in both negative and positive ion modes with a Bruker-Reflex II mass spectrometer (Bruker–Daltonics) with delayed ion extraction at an acceleration voltage of 20 kV. The dried samples were dissolved in distilled water at a concentration 10–20 μg μL^{-1} and 1 μL aliquot was mixed with 1 μL 0.5 M matrix soln of recrystallised 2,5-dihydroxybenzoic acid (Aldrich) in methanol. A 0.5 μL aliquot was deposited on metallic sample holder and analysed immediately after drying in a stream of air. The mass scale was calibrated externally with similar compounds of known chemical structure. The spectra shown represent the average of at least 50 single laser shot analyses.

O-deacylated LPS of the Δ*algC* mutants and core oligosaccharides isolated by HPAEC were analysed in the negative ion mode using Fourier-transform ion cyclotron resonance ESIMS on an Apex II instrument (Bruker–Daltonics) equipped with a 7 Tesla actively shielded magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Samples were dissolved at a concentration ~20 μg μL^{-1} in 50:50:0.001 2-propanol–water–triethylamine and sprayed at a flow rate 2 μL min^{-1}. Capillary entrance voltage was set to 3.8 kV and dry gas temperature to 150 °C. CSD was induced by increasing the capillary exit voltage from − 100 to − 350 V.

3.9. NMR spectroscopy

Samples were lyophilised three times from ~1 mL D_2O (99.6% D; Cambridge Isotope Laboratories) and measured in 3-mm diameter high precision NMR glass sample tubes (Promochem) in ~0.2 mL D_2O (99.996% D; Cambridge Isotope Laboratories). Spectra for the *O*-polysaccharides were measured at 300 and 340 K and for the core oligosaccharides at 300 K. Chemical shifts were referenced to separately measured acetone (δ_H 2.225; δ_C 31.45 ppm) and aq 85% phosphoric acid (δ_P 0.00 ppm). Standard Bruker XWINNMR 2.6 software was used to acquire and process the NMR data. Proton-decoupled ^{13}C NMR spectra were recorded with an Avance DPX-360 spectrometer (Bruker) at 90.6 MHz. ^{1}H NMR spectra with and without water suppression, proton-decoupled ^{31}P NMR spectra, 2D homonuclear ^{1}H,^{1}H NMR (COSY, TOCSY with mixing time 100 ms, and NOESY with mixing time 300 ms), heteronuclear H-detected ^{1}H,^{13}C NMR (HMQC, HMBC) and ^{1}H,^{31}P NMR (HMQC, HMQC-TOCSY) spectra were run on an Avance DRX-600 spectrometer (Bruker) at 600.01 MHz (^{1}H), 150.9 MHz (^{13}C), and 242.8 MHz (^{31}P). H-detected ^{1}H,^{31}P HMQC-TOCSY experiments were performed using the published procedure[47] adapted to the phosphorus frequency. The 2D spectra were acquired with 384 experiments each with 2048 data points in the *F*2 dimension and appropriate number of scans. After zero-filling in the *F*1 dimension and sinusoidal multiplication in both dimensions, the data were Fourier-transformed to the ^{1}H, ^{13}C, and ^{31}P NMR spectra with a range of 4000, 30,000, and 10,000, respectively.

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