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Note

Structure of the core part of the lipopolysaccharides from *Proteus penneri* strains 7, 8, 14, 15, and 21

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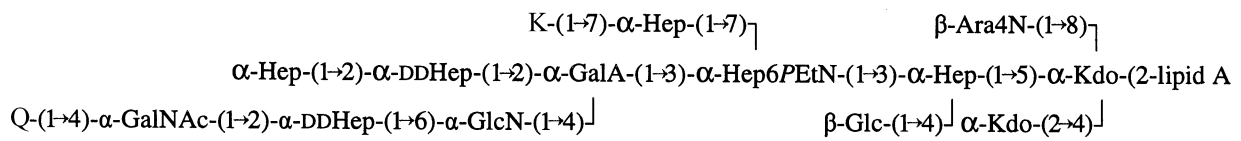
Abstract

The core–lipid A region of the lipopolysaccharides from *Proteus penneri* strains 7, 8, 14, 15, and 21 was studied using NMR spectroscopy, ESI MS, and chemical analysis after alkaline deacylation, deamination, and mild-acid hydrolysis of the lipopolysaccharides. The following general structure of the major core oligosaccharides is proposed:



where all sugars are in the pyranose form and have the D configuration unless otherwise stated, Hep and DDHep = L-*glycero*- and D-*glycero*-D-*manno*-heptose, respectively, K = H, and Q = H in strain 8 or α-Glc in strains 7, 14, 15, and 21. In addition, several minor structural variants are present, including those lacking Ara4N in strains 7 and 15 and having the α-GlcN residue *N*-acylated to a various degree with glycine in strains 7, 8, 14, and 21. In strain 14, there are also core oligosaccharides with K = amide of β-D-Gal*p*A with putrescine, spermidine, or 4-azaheptane-1,7-diamine; remarkably, these structural variants lack either the *P*EtN group or the α-Hep-(1 → 2)-α-DDHep disaccharide fragment at α-D-Gal*p*A. While structural features of the inner core part are shared by *Proteus* strains studied earlier, the outermost Q-(1 → 4)-α-GalNAc-(1 → 2)-α-DDHep-(1 → 6)-α-GlcN oligosaccharide unit has not been hitherto reported.

Keywords: Lipopolysaccharide; *Proteus*; *Proteus penneri*; Core structure

1. Introduction

Gram-negative bacteria of the genus *Proteus* are opportunistic pathogens causing nosocomial and urinary tract infections.[1] The genus *Proteus* consists of five species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri*, and *P. myxofaciens*, and three unnamed genomospecies 3, 4, and 5.[2,3] Lipopolysaccharide (LPS) is the major component of the outer membrane and considered as an important virulence factor of *Proteus* bacteria.[4] LPS comprises three regions: lipid A, the O-specific polysaccharide (O-antigen), and the core, which is a non-repetitive oligosaccharide intervening the O-specific polysaccharide and lipid A. The majority of *Proteus* O-antigens, which serve as the basis for classification of

Abbreviations: LPS, lipopolysaccharide; Hep, L-*glycero*-D-*manno*-heptose; DDHep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; *P*EtN, 2-aminoethyl phosphate; Ara4N, 4-amino-4-deoxy-L-arabinose; anhMan, 2,5-anhydromannose; ΔGalA, 4-deoxy-β-L-*threo*-hex-4-enuronic acid; GalAPu, GalASp, and GalAT, amides of GalA with butane-1,4-diamine (putrescine), 4-azaoctane-1,8-diamine (spermidine), and 4-azaheptane-1,7-diamine, respectively; HPAEC, high-performance anion-exchange chromatography.

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strains to O-serogroups, have been structurally characterized.[5] The core region has been investigated in a number of *Proteus* strains and found to consist of a structurally conserved part next to lipid A and a variable outermost part, which in many cases is O-serogroup-dependent.[6–14] Several structural variants of the core are present in each strain, which differ in the presence or absence of some sugar and non-sugar substituents.

Now we present results of structural analysis of five strains of *P. penneri*, four of which have the same carbohydrate backbone in the main structural variant and the fifth has a truncated structure. Structures of the O-specific polysaccharides of *P. penneri* 8 (serogroup O67),[15] 14,[16] 15,[17] and 21 (identical to that of *P. penneri* 52, serogroup O61[18]) have been established. LPS from strain 7 has no O-polysaccharide chain.

2. Results and discussion

Strong alkaline degradation of the LPS was applied to fully deacylate the lipid A moiety and to obtain phosphorylated core–lipid A carbohydrate backbone.[19] In *Proteus* LPS, the alkaline deacylation is accompanied by β elimination of the glycosyl substituent from position 4 of an α-GalA residue.[6,7,9–14] The products obtained from the LPSs of five *P. penneri* strains studied were analyzed by HPAEC and ESI MS using published data[6,9,11] and found to be complex mixtures of related compounds of the general formula **1** containing a 4,5-unsaturated hexuronic acid (ΔGalA) derived from 4-substituted GalA H (Fig. 1). GlcN B of the lipid A backbone was not phosphorylated, most likely, owing to elimination of β-Ara4N-1-*P* under the alkaline conditions.[10]

The mixture from each strain was separated by preparative HPAEC to give compounds **1a**–**1e** (Fig. 1, Table 1). The major oligosaccharide **1a** was obtained from all strains and found to be identical by retention time in HPAEC, ESI MS, and NMR spectroscopy data to the compound isolated previously.[6,9,11] As inferred from ESI MS data (Fig. 2), minor oligosaccharides **1b**–**1e** differed from **1a** in the absence of β-Ara4N Z at Kdo C (**1b**) or the presence of β-GalA K at Hep G (**1c**–**1e**). As compared to **1a**, the latter variant lacked either *P*EtN at Hep F (**1e**) or an α-Hep-(1→2)-α-DDHep disaccharide at GalA H (**1d**) or was devoid of both these substituents (**1c**). Structures of **1b**–**1e** were

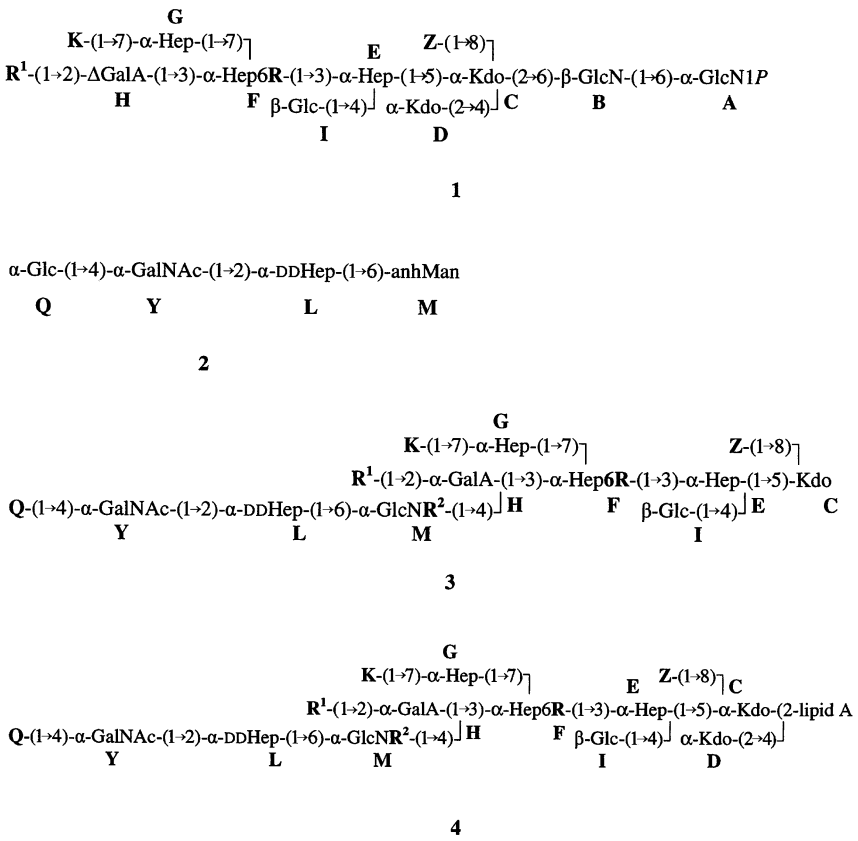

Fig. 1. Structures of the isolated oligosaccharides and proposed structure of the core–lipid A carbohydrate backbone of the LPS from *P. penneri* 7, 8, 14, 15, 21, and 25. All sugars are in the pyranose form except anhMan in **2** and Kdo in **3**. For variable substituents see Tables 1 and 2.

Table 1
Variable substituents in oligosaccharides **1a**–**e** isolated by alkaline degradation of *P. penneri* LPSs

Compound	K	R	R[1]	Z	Derived from *P. penneri* strain
1a	H	*P*EtN	α-Hep-(1→2)-α-DDHep X T	β-Ara4N	7, 8, 14, 15, 21
1b	H	*P*EtN	α-Hep-(1→2)-α-DDHep	H	7, 15, 21
1c	β-GalA	H	H	β-Ara4N	7, 8, 14, 15, 21
1d	β-GalA	*P*EtN	H	β-Ara4N	14
1e	β-GalA	H	α-Hep-(1→2)-α-DDHep	β-Ara4N	14

confirmed by NMR spectroscopy using for comparison data of **1a** and previously published data on related *Proteus* core oligosaccharides containing β-GalA K at Hep G.[6,9,11] Like **1a**, compound **1c** was obtained from all strains, whereas the other minor products were derived only from strain 14 (**1d** and **1e**) or strains 7, 15, and 21 (**1b**).

Deamination of the LPSs from *P. penneri* 7 and 21 resulted in cleavage of the linkage between GlcN M and GalA H to give oligosaccharide **2** with 2,5-anhydromannose (anhMan) at the reducing end (Fig. 1), which was purified by reverse-phase HPLC. The structure of **2** was established by sugar analysis, including GLC of the acetylated alditols and 2-butyl glycosides for determination of the absolute configurations, methylation analysis, and NMR spectroscopy after full assignment of the ^{1}H and ^{13}C NMR spectra (Table 3). In addition to anhMan, **2** was found to contain α-Glc Q, α-GalNAc Y, and D-*glycero*-α-D-*manno*-heptose (α-DDHep) L. The spin systems of the monosaccharides were identified based on the H, H coupling constant values determined from the ^{1}H NMR spectrum, and α-DDHep L was distinguished from the L-*glycero*-D-*manno* isomer (Hep) by the C-7 chemical shift.[6] Linkage and sequence analysis of **2** was performed using NOESY and HMBC experiments, which showed the following interresidue correlations: Q H-1,Y H-4 (strong), Q H-1,Y H-6a (medium), Q H-1,Y H-6b (weak), Y H-1,L H-2 (strong), Y H-1,L H-3 (weak), L H-1,M H-6a (strong), L H-1,M H-6b (weak), L H-1,Y H-5 (medium) in the NOESY spectrum, and Q H-1,Y C-4, Y H-1,L C-2, and L H-1,M C-6 in the HMBC spectrum.

Mild-acid hydrolysis of the LPSs with aq 2% HOAc followed by fractionation of the products by sequential GPC and anion-exchange HPLC gave oligosaccharides **3** (Fig. 1, Table 2). The whole mixture of the core products as obtained by GPC were analyzed by ESI MS (Figs. 3 and 4). Oligosaccharide pairs **3a**, **3b** (major) and **3c**, **3d** (minor) were obtained from strain 7 and some of them also from strains 14, 15, and 21 with **3a** as the major compound (Table 2). Another pair, **3e**, **3f** with a predominance of **3e**, was isolated from strain 8. These products differed in the presence (**3a**, **3c**, and **3e**) or absence (**3b**, **3d**, and **3f**) of the glycine residue as the *N*-acyl substituent of GlcN M. Compounds **3e** and **3f** lacked a hexose residue (Glc Q, see below) and compounds **3c** and **3d** the Ara4N residue **Z** as compared to compounds **3a** and **3b**.

In addition to **3a** and **3b**, the LPS of *P. penneri* 14 afforded oligosaccharides **3g**–**3p** as minor compounds present in quantity of ~10% relative to **3a**, **3b**. They contained an additional β-GalA residue K, which was

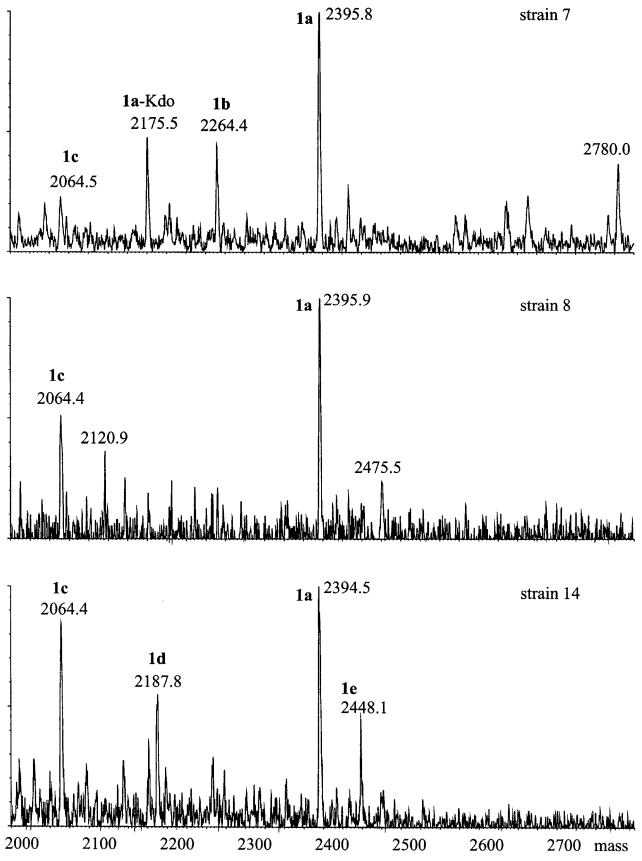

Fig. 2. Charge deconvoluted ESI MS spectra of mixtures of alkaline degradation products from *P. penneri* strains 7, 8, and 14.

Table 2
Variable substituents in oligosaccharides **3a**–**p** isolated by mild-acid degradation of *P. penneri* LPSs

Compound	R	R^1	R^2	Q	K	Z	Derived from *P. penneri* strain
3a	*P*EtN	α-Hep-(1→2)-α-DDHep X T	H	α-Glc	H	β-Ara4N	7, 14, 15, 21
3b	*P*EtN	α-Hep-(1→2)-α-DDHep	Gly	α-Glc	H	β-Ara4N	7, 14, 21
3c	*P*EtN	α-Hep-(1→2)-α-DDHep	H	α-Glc	H	H	7, 15
3d	*P*EtN	α-Hep-(1→2)-α-DDHep	Gly	α-Glc	H	H	7
3e	*P*EtN	α-Hep-(1→2)-α-DDHep	H	H	H	β-Ara4N	8
3f	*P*EtN	α-Hep-(1→2)-α-DDHep	Gly	H	H	β-Ara4N	8
3g	H	α-Hep-(1→2)-α-DDHep	H	α-Glc	β-GalAPu	β-Ara4N	14
3h	H	α-Hep-(1→2)-α-DDHep	H	H	β-GalAPu	β-Ara4N	14
3i	H	α-Hep-(1→2)-α-DDHep	H	α-Glc	β-GalASp	β-Ara4N	14
3j	H	α-Hep-(1→2)-α-DDHep	H	H	β-GalASp	β-Ara4N	14
3k	H	α-Hep-(1→2)-α-DDHep	H	α-Glc	β-GalAT	β-Ara4N	14
3l	*P*EtN	H	H	α-Glc	β-GalAPu	β-Ara4N	14
3m	*P*EtN	H	H	H	β-GalAPu	β-Ara4N	14
3n	*P*EtN	H	H	α-Glc	β-GalASp	β-Ara4N	14
3o	*P*EtN	H	H	H	β-GalASp	β-Ara4N	14
3p	*P*EtN	H	H	α-Glc	β-GalAT	β-Ara4N	14

amidated with butane-1,4-diamine (putrescine), 4-aza-octane-1,8-diamine (spermidine), or 4-azaheptane-1,7-diamine. Compounds **3g**–**3p** were separated by cation-exchange HPLC into two groups, one containing putrescine (**3g**, **3h**, **3l**, and **3m**) and the other spermidine or 4-azaheptane-1,7-diamine (**3i**–**3k**, **3n**–**3p**).

Structures of the oligosaccharides **3** were determined by sugar and methylation analysis along with ESI MS and NMR spectroscopy (Table 3). Structural determination of the common core fragment containing residues C to H (including the *P*EtN-bearing Hep F), M, T, X, and Z on the basis of NMR data have been described earlier in studies of similar oligosaccharides isolated by mild-acid hydrolysis of the LPS from some other *Proteus* strains.[6,9,11] In oligosaccharides **3a** and **3b**, a trisaccharide fragment consisting of residues L, Y, and Q was identified, which had the ^{1}H and ^{13}C NMR chemical shifts (Table 3) and showed the NOESY and HMBC patterns similar to those of oligosacchride **2**. In accordance with the ESI MS data (Fig. 3), the NMR spectra of compounds **3e** and **3f** obtained from *P. penneri* 8 lacked the signals for α-Glc Q.

In compounds **3a** and **3e**, GlcN M had a free amino group as followed from the ESI MS data (Fig. 3) and a relatively high-field position of the H-2 signal at δ 3.31 in the ^{1}H NMR spectrum. In compounds **3b** and **3f**, the amino group was acylated, which caused a low-field shift of the H-2 signal of GlcN M to δ 3.94 ppm. The acyl group was identified as a glycine residue by correlation of the H-2 signals at δ 3.79 and 4.02 with the C-2 signal at δ 41.9 in HMQC and the C-1 signal at δ 168.6 in HMBC experiments. The C-1 of glycine gave also a long-range (HMBC) correlation with H-2 of GlcN M.

The NMR spectra of the compounds **3g**–**3p** from the LPS of *P. penneri* 14 contained additional signals for β-GalA K (Table 2). A NOE between H-1 of β-GalA K and H-7 of Hep G indicated the site of attachment of the residue K. The NMR spectra revealed also signals of putrescine (in **3g**, **3h**, **3l**, and **3m**), spermidine (in **3i**, **3j**, **3n**, and **3o**), and 4-azaheptane-1,7-diamine (in **3k** and **3p**). The amines were identified also by HPLC after

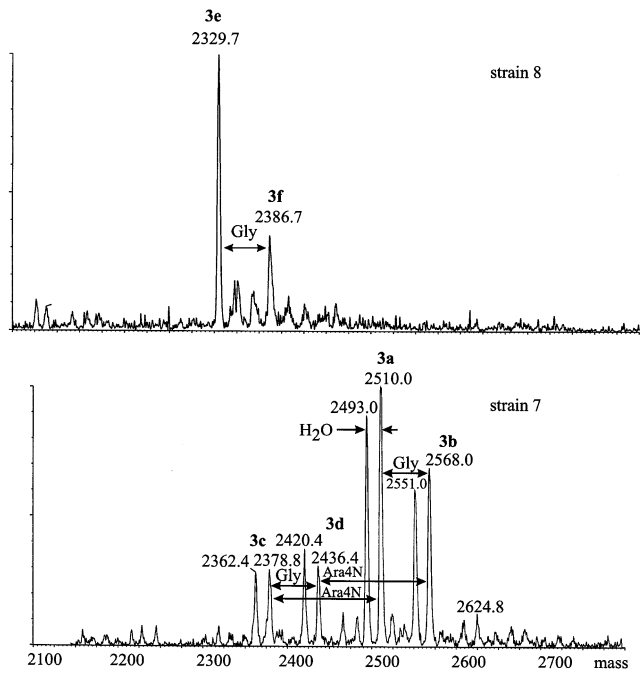

Fig. 3. Charge deconvoluted ESI MS spectra of the neutral core fraction from *P. penneri* 7 and 8. Each compound from strain 7 gave two peaks owing to partial loss of water from Kdo.

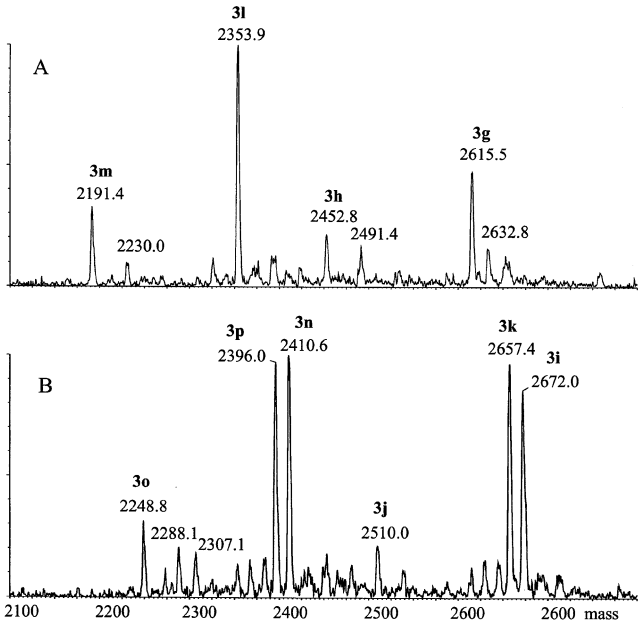

Fig. 4. Charge deconvoluted ESI MS spectra of the basic core fractions from *P. penneri* 14 LPS. (A) Compounds with putescine; (B) compounds with spermidine and 4-azaheptane-1,7-diamine.

acid hydrolysis as described.[20] Their attachment via the NH_2-1 group to the carboxyl group was confirmed by an HMBC correlation between H-1 of the amine and C-6 of β-GalA K. The NMR spectroscopic pattern of the compounds **3g**–**3p** was similar to that reported for other *Proteus* core products containing amides of β-GalA.[12] Compounds **3g**–**3p** occurred as two major variants: one with the α-Hep-(1→2)-α-DDHep (X–T) disaccharide fragment at position 2 of α-GalA H and without *P*EtN at position 6 of Hep F (**3g**–**3k**) and the other without X–T and with *P*EtN (**3l**–**3p**) (Table 2). These variants could be readily distinguished by ESI MS data and by the presence of the corresponding signals in the NMR spectra (for assignment of the signals from X–T see published data[9]).

Comparison of the structures of the oligosaccharides (Fig. 1, Tables 1 and 2) showed the following correspondence between the alkaline degradation products **1** and mild-acid hydrolysis products **3**:

1a⇔**3a**, **3b**, **3e**, **3f**;

1b⇔**3c**, **3d**;

1d⇔**3l**–**3p**; and

1e⇔**3g**–**3k**.

Table 3
Selected ^{1}H and ^{13}C NMR chemical shifts of oligosaccharides **2** and **3** (δ, ppm)

Residue, compound	Nucleus	1	2	3	4	5	6(a)	7a(6b)	7b
α-GalA H,	^{1}H	5.46	4.05	4.23	4.43	4.49			
3a and **3e**	^{13}C	99.3	73.0	67.8	80.8	72.3	175.9		
α-GalA H,	^{1}H	5.46	4.05	4.19	4.38	4.43			
3b and **3f**	^{13}C	99.3	73.0	67.8	81.4	72.7	175.4		
anhMan M,	^{1}H	5.07	3.74	4.18	4.11	4.02	3.67	3.83	
2	^{13}C	90.8	85.4	78.5	77.8	82.2	67.4		
α-GlcN M,	^{1}H	5.17	3.31	3.88	3.61	4.43	3.61	4.05	
3a and **3e**	^{13}C	97.5	55.2	70.8	70.3	72.6	66.0		
α-GlcN M,	^{1}H	4.94	3.94	3.74	3.61	4.43	3.61	4.01	
3b and **3f**	^{13}C	100.5	54.8	72.3	70.3	72.6	66.0		
α-DDHep L,	^{1}H	5.17	3.94	3.95	3.83	3.72	4.05	3.71	3.83
2	^{13}C	99.2	80.6	71.6	68.5	74.4	72.9	63.0	
α-DDHep L,	^{1}H	5.14	3.94	3.93	3.85	3.72			
3	^{13}C	99.2	80.5	71.7	67.3	74.1			
α-GalNAc Y,	^{1}H	5.11	4.23	4.03	4.09	4.10	3.83	3.89	
2	^{13}C	100.9	51.6	68.3	79.5	73.1	61.6		
α-GalNAc Y,	^{1}H	5.12	4.23	4.04	4.11	4.11	3.83	3.90	
3a	^{13}C	101.0	51.5	68.2	79.5	73.1	61.5		
α-GalNAc Y,	^{1}H	5.07	4.16	3.93	4.00	4.05	3.75	3.75	
3e	^{13}C	100.4	51.4	68.7	69.8	72.8	62.5		
α-Glc Q,	^{1}H	4.95	3.54	3.79	3.46	4.06	3.72	3.79	
2	^{13}C	101.6	72.9	73.8	70.3	73.1	61.1		
α-Glc Q,	^{1}H	4.96	3.55	3.80	3.46	4.06	3.73	3.80	
3	^{13}C	101.4	72.9	73.9	70.3	73.1	61.1		

The chemical shifts for Gly in compounds **3b** and **3f** are δ_H 3.79 and 4.02 (H-2a,2b), δ_C 168.6 (C-1) and 41.9 (C-2).

Table 4
Occurrence of various core variants in *P. penneri* LPSs

P. penneri strain	R	R^1	R^2	Q	Z	K
7	*P*EtN	α-Hep-(1→2)-α-DDHep	H or Gly (~3:1)	α-Glc	H	H
7, 14, 21	*P*EtN	α-Hep-(1→2)-α-DDHep	H or Gly (~3:1)	α-Glc	Ara4N	H
8	*P*EtN	α-Hep-(1→2)-α-DDHep	H or Gly (~9:1)	H	Ara4N	H
14	H	α-Hep-(1→2)-α-DDHep	H	α-Glc or H	Ara4N	β-GalAPu β-GalASp β-GalAT
14	*P*EtN	H	H	α-Glc or H	Ara4N	β-GalAPu β-GalASp β-GalAT
15	*P*EtN	α-Hep-(1→2)-α-DDHep	H	α-Glc	Ara4N or H	H

For the core structure and position of the variable substituents see structure **4** in Fig. 1.

However, no oligosaccharide **3** corresponded to the compound **1c**, which lacked both *P*EtN substituent at O-6 of Hep F and α-Hep-(1→2)-α-DDHep and which was obtained by alkaline degradation of all LPS studied. Indeed, all core structural variants from strains 7, 8, 15, and 21 contained the X–T fragment, and although the variants that lacked this fragment were found in strain 14, they all contained the *P*EtN group. Therefore, most likely, **1c** is an experimental artefact.

In conclusion, the generalized core structure **4** of the LPSs studied is shown in Fig. 1. All core variants contain common structural elements but also have a number of distinctions (Table 4). The content of each structural variant varies significantly from strain to strain. The major variants contain a characteristic α-Hep-(1→2)-α-DDHep disaccharide fragment X–T, found in all *P. penneri* strains analyzed so far,[6,9,11] but this fragment was absent from some minor structural variants. The LPS core of *P. penneri* 14 includes a low amount of β-GalA K amidated with spermidine, putrescine, or 4-azaheptane-1,7-diamine, from which the last amine has been found for the first time in LPS. Remarkably, the amidated β-GalA-containing structural variants lack either the *P*EtN substituent at O-6 of Hep **F** or the α-Hep-(1→2)-α-DDHep fragment X–T, which suggests a mutual biosynthetic dependence of the three components.

3. Experimental

Bacteria.—These were cultivated and LPS was isolated as described.[9]

NMR spectroscopy and general methods.—^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 500 spectrometer in D_2O at 25 °C with acetone standard (2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C) using standard pulse sequences gCOSY, TOCSY (mixing time 120 ms), NOESY (mixing time 250 ms), HSQC, gHMBC (optimized for 5 Hz long-range coupling constant), gHSQC-TOCSY (mixing time 80 ms). ^{31}P and ^{1}H–^{31}P HMQC (optimized for 8 Hz ^{31}P–^{1}H coupling constant) spectra were recorded on Varian Inova 400 spectrometer. Electrospray mass spectra were obtained on Micromass Quattro spectrometer in negative mode, with direct injection in 50% MeCN with 0.2% HCO_2H at flow rate 15 mkl/min. GLC, GLC–MS, methylation, monosaccharide analysis were performed as previously described.[12]

Preparation of oligosaccharides **1**.—LPS from each strain (200 mg) was heated at 100 °C for 4 h in 4 M NaOH (4 mL), cooled, and 2 M HCl (7.5 mL) was added. The precipitate was removed by centrifugation and the substance desalted on a Sephadex G50 column. The oligosaccharide fraction was separated on a Carbopac PA100 (250 × 9 mm) column using a gradient of 20–80% of 1M NaOAc in 0.1 M NaOH at 3 mL/min over 1 h to give after desalting oligosaccharides **1**.

Preparation of oligosaccharide **2**.—LPS (200 mg) was dissolved in water (20 mL), 200 mg of $NaNO_2$ and 1 mL of AcOH were added. After 3 h at 25 °C, the lipid containing part of LPS was removed by ultracentrifugation (55,000 rpm), and the supernatant was separated by gel chromatography on Sephadex G50S. Oligosaccharides were partially coeluted with salt and were additionally desalted on a Sephadex G15 column, and then purified by reverse-phase HPLC on an Aqua C18 column (Phenomenex, 1 × 25 cm) in water with UV detection at 220 nm, yield 8 mg.

Preparation of oligosaccharides **3**.—LPS (200 mg) was hydrolyzed with 2% AcOH (100 °C, 4 h). The resulting precipitate was removed by centrifugation,

and the supernatant separated on a Sephadex G50 SF gel (Pharmacia) column (2.5×80) using pyridine–AcOH buffer (4 mL of pyridine and 10 mL of AcOH in 1 L of water) monitoring by Waters differential refractometer. Core fractions were then separated by anion-exchange chromatography on a Hitrap Q (5 mL, Pharmacia–Amersham) column in a gradient of 0–1 M NaCl to give neutral and acidic fractions. A neutral fraction, obtained from strain 14, was further separated on a Hitrap S column in the same gradient.

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