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Note

Structure of the O-specific polysaccharide of *Providencia rustigianii* O14 containing N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine

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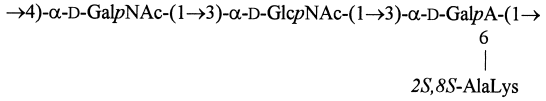
Abstract

The O-specific polysaccharide of *Providencia rustigianii* O14 was obtained by mild acid degradation of the LPS and studied by chemical methods and NMR spectroscopy, including 2D ^{1}H,^{1}H COSY, TOCSY, NOESY, and ^{1}H,^{13}C HSQC experiments. The polysaccharide was found to contain N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine ('alaninolysine', 2*S*,8*S*-AlaLys). The amino acid component was isolated by acid hydrolysis and identified by ^{13}C NMR spectroscopy and specific optical rotation, using synthetic diastereomers for comparison. The following structure of the trisaccharide repeating unit of the polysaccharide was established:



Anti-*P. rustigianii* O14 serum was found to cross-react with O-specific polysaccharides of *Providencia* and *Proteus* strains that contains amides of uronic acid with N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine and L-lysine.

Keywords: Lipopolysaccharide; O-Specific polysaccharide; Bacterial polysaccharide structures; N^{α}-(D-galacturonoyl)-N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine; *Providencia rustigianii*

Bacteria of the genus *Providencia* are serologically related to *Escherichia coli*, *Proteus*, *Morganella*, *Salmonella* and *Shigella*.[1] Currently, the genus consists of five species: *P. alcalifaciens*, *P. heimbachae*, *P. rettgerii*, *P. rustigianii* and *P. stuartii*. *P. rustigianii* (formerly known as *Providencia alcalifaciens* biogroup 3) is a facultative bacterial pathogen, which cause intestinal infections particularly in children.[2] *P. alcalifaciens* is more frequently isolated from the stool of adults with diarrhoea who had travelled abroad.[3,4] The bacteria are able to invade intestinal mucosa and other cell types.

Like other bacteria from the tribe *Proteae*, which includes also the genera *Proteus* and *Morganella*, bacteria *Providencia* have an O-antigen, a flagellar H-antigen and a capsular K-antigen, which form the basis for the serological classification. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 62 O-serogroups and 30 H-serogroups.[5,6] The O-antigenic specificity of Gram-negative bacteria is defined by the structure of the O-antigen, which represents a polysaccharide chain (O-specific polysaccharide, OPS) of the lipopolysaccharide (LPS), one of the major components of the outer membrane of the cell wall. Among bacteria of the *Enterobacteriaceae* family, the genus *Providencia* is one of the least studied in respect to the LPS structure. Recently, the structures of the OPS of *P. alcalifaciens* O5,[7] O7,[8] O16[9] and O23[10] have

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been established. Now we report on the structure and serological properties of the O-antigen of *P. rustigianii* O14.

The OPS was obtained by mild acid degradation of the LPS, isolated from bacterial cells by the phenol–water procedure,[11] followed by GPC on Sephadex G-50. Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis and determination of the absolute configurations of the monosaccharides by GLC of the acetylated (+)-2-octyl glycosides[12,13] revealed D-GalN and D-GlcN in the ratio 1.8:1. The presence of GalA was demonstrated by analysis of the OPS hydrolysate using a sugar analyser, and its absolute configuration was determined by analysis of the glycosylation effects in the ^{13}C NMR spectrum of the OPS (see below).

The ^{13}C NMR spectrum of the OPS (Fig. 1) showed signals for three sugar residues, including those for three anomeric carbons at δ 100.5, 98.3 and 95.5, two nitrogen-bearing carbons at δ 50.2 and 52.6 (C-2 of GalN and GlcN), two $HOCH_2$–C groups at δ 59.8 and 60.5 and other sugar carbons in the region δ 67.1–77.2. The spectrum contained also signals of *N*-acetyl groups at δ 22.5 (2C) and N^{ε}-(1-carboxyethyl)lysine ('alaninolysine', AlaLys) at δ 15.5, 22.5, 26.0, 32.0, 46.5, 55.0 and 58.3 (compare published data[10,14]). There were five signals of carbonyl groups at δ 170.7–179.0 belonging to C-6 of GalA, two *N*-acetyl groups, C-1 and C-7 of AlaLys. The absence from the spectrum of any signals in the region 82–88 ppm characteristic of furanosides[15] indicated that all monosaccharides are in the pyranose form. Accordingly, the ^1H NMR spectrum of the OPS showed, *inter alia*, signals for three anomeric protons at δ 5.42, 5.17 and 5.08 and two *N*-acetyl groups at δ 2.03 and 2.07 (both singlets).

The amino acid **1** was isolated from the OPS hydrolysate by GPC on TSK HW-40. The ^1H and ^{13}C NMR spectra of **1** showed signals for the alanine and lysine moieties with signals for C-6 of lysine and C-2 of alanine shifted downfield to δ 46.5 and 58.3, as compared with their positions at δ 40.6 and 51.6, respectively, in the corresponding free amino acid. These data confirmed that **1** is N^{ε}-(1-carboxyethyl)lysine.

A positive optical rotation value of **1**, $[\alpha]_D$ +9.5° (*c* 0.14, water), showed that the lysine moiety has the L configuration {compare published data[16] $[\alpha]_D$ +9.7 and +11.6° for N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine (2*S*,8*R*-AlaLys) and N^{ε}-[(*S*)-1-carboxyethyl)-L-lysine (2*S*,8*S*-AlaLys), respectively}. The *S* configuration of the second anomeric center in **1** was determined by ^{13}C NMR spectroscopy as described,[10,17] using authentic samples of 2*S*,8*R*-AlaLys and 2*S*,8*S*-AlaLys for comparison. Therefore, **1** is N^{ε}-[(*S*)-1-carboxyethyl)-L-lysine (Fig. 2).

The ^1H and ^{13}C NMR spectra of the OPS were assigned using 2D COSY, TOCSY, NOESY, and H-detected ^1H,^{13}C HSQC experiments (Tables 1 and 2). The TOCSY spectrum demonstrated following correlations: H-1/H-2 and H-1/H-3 for GalNAc, H-1/H-2–H-5 for GalA, and H-1/H-2–H-6 for GlcNAc. The COSY spectrum showed correlations between the neighbouring protons within each spin system, but a few signals could not be reliably assigned because of their coinci-

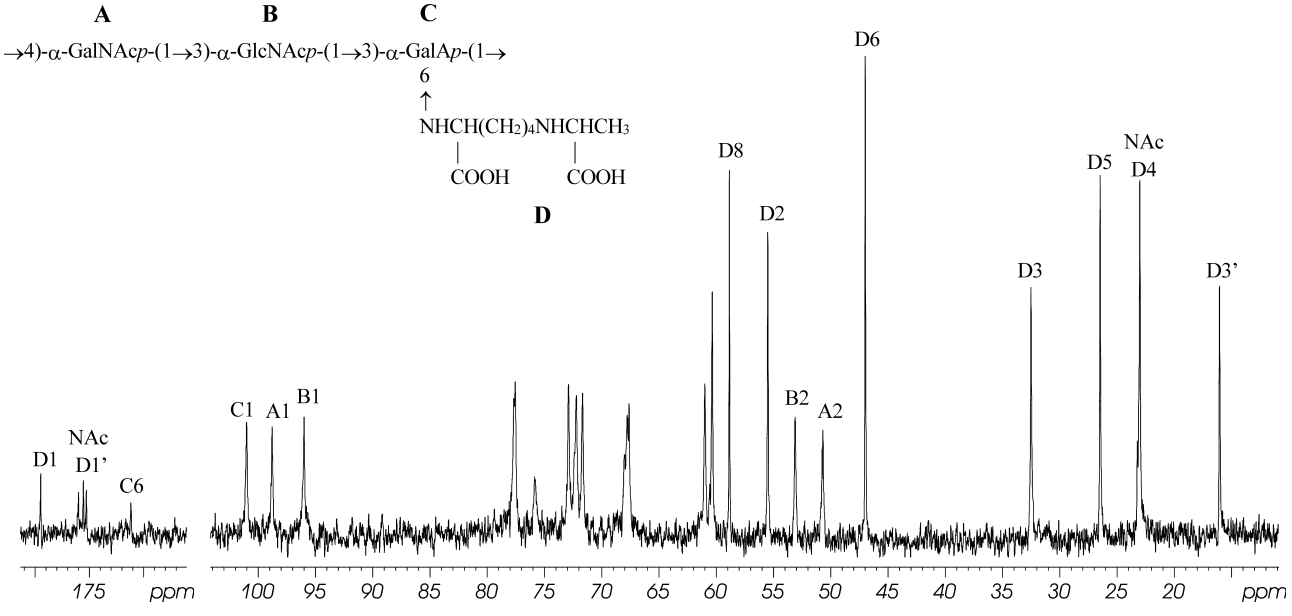

Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide of *P. rustigianii* O14. Arabic numerals refer to carbons of sugar and amino acid residues denoted by letters as shown in the structure at the top. Carbon numeration for the amino acid (D) is shown in Fig. 2.

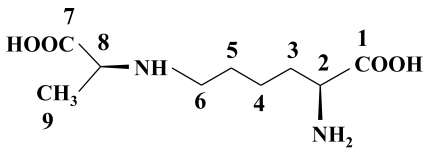

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Fig. 2. Structure and carbon numeration of N^{ε}-[(*S*)-1-carboxyethyl)-L-lysine (**1**).

dences. To overcome this difficulty, two-dimensional H-detected ^{1}H, ^{13}C HSQC experiment was used. The spin systems of GalNAc and GlcNAc residues were distinguished by correlations of protons at carbons bearing nitrogens (H-2) to the corresponding carbons (C-2) at 4.21/50.2 and 4.10/52.6, respectively.

The α configuration of all glycosidic linkages was determined by the position of the H-1 signals at δ 5.42, 5.17 and 5.08 and $J_{1,2}$ coupling constant values < 3 Hz. The presence in the NOESY spectrum (Fig. 3) of strong intraresidue H-1,H-2 cross-peaks, but no H-1,H-3 or H-1,H-5 cross-peaks, for GalNAc, GlcNAc and GalA confirmed the sugar residues are α-linked.

The NOESY spectrum of the OPS (Fig. 3) showed strong interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: GalNAc H-1, GlcNAc H-3 at δ 5.42/3.97, GlcNAc H-1, GalA H-3 at δ 5.08/4.08 and GalA H-1,GalNAc H-4 at δ 5.17/4.19. These data defined the glycosylation pattern and the monosaccharide sequence in the repeating unit. The modes of substitution of the monosaccharides were confirmed by down-field displacement of the signals for linkage carbons to δ 75.4–77.2 as compared with their positions in non-substituted monosaccharides.[18] Methylation analysis of the OPS revealed a derivative from the 3-substituted GlcNAc residue, whereas no derivative from the 4-substituted GalNAc was detected evidently owing to its retention during acid hydrolysis as a methylated GalA → GalN disaccharide.

The D configuration of GalA was inferred based on the C-1 chemical shift of α-GlcNAc, which substitutes GalA at position 3. The value of δ 95.5, and the corresponding effect on C-1 of ~ 3 ppm as compared with the C-1 chemical shift in the free monosaccharide,[18] demonstrated the same absolute configuration of GlcNAc and GalA (in case of their different configurations the effect on C-1 would be > 6 ppm[19]). The position of AlaLys at C-6 of GalA (Fig. 4) followed from its resonance at δ 170.7 that is characteristic for hexuronamides.[10]

Therefore, it was concluded that the repeating unit of the O-specific polysaccharide of *P. rustigianii* O14 has the structure shown in Fig. 5. This is the first bacterial polysaccharide reported to contain an amide of a hexuronic acid with N^{ε}-[(*S*)-1-carboxyethyl)-L-lysine. Amides of D-GlcA and D-GalA with a diastereomeric amino acid N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine (Fig. 4) has been previously found in the O-specific polysaccharide of *P. alcalifaciens* O23[10] and those of taxonomi-

Table 1
^{1}H NMR chemical shifts of the O-specific polysaccharide (δ, ppm)

Sugar residue	H-1 H-1	H-2 H-2	H-3 H-3	H-4 H-4	H-5 H-5	H-6a H-6	H-6b H-7	H-8	H-9
→4)-α-D-Gal*p*NAc-(1→	5.42	4.21	3.93	4.19	3.94	3.86	3.86		
→3)-α-D-Glc*p*NAc-(1→	5.08	4.10	3.97	3.74	3.96	3.85	3.80		
→3)-α-D-Gal*p*A-(1→	5.17	4.00	4.08	4.46	4.87				
AlaLys		4.25	1.85 [a]	1.40	1.71	3.02		3.64	1.47

Signals for NAc are at δ 2.03 and 2.07.
[a] H-3a; H-3b at δ 1.72.

Table 2
^{13}C NMR chemical shifts of the O-specific polysaccharide (δ, ppm)

Sugar residue	C-1 C-1	C-2 C-2	C-3 C-3	C-4 C-4	C-5 C-5	C-6 C-6	C-7	C-8	C-9
→4)-α-D-Gal*p*NAc-(1→	98.3	50.2	67.2	77.1	71.7	59.8			
→3)-α-D-Glc*p*NAc-(1→	95.5	52.6	77.2	71.2	72.4	60.5			
→3)-α-D-Gal*p*A-(1→	100.5	67.4	75.4	67.1	71.7	170.7			
AlaLys	179.0	55.0	32.0	22.5	26.0	46.5	175.1 [a]	58.3	15.5

Signals for NAc are at δ 22.5 (2C, Me), 174.8 [a] and 175.5 [a] (CO).
[a] Assignments could be interchanged.

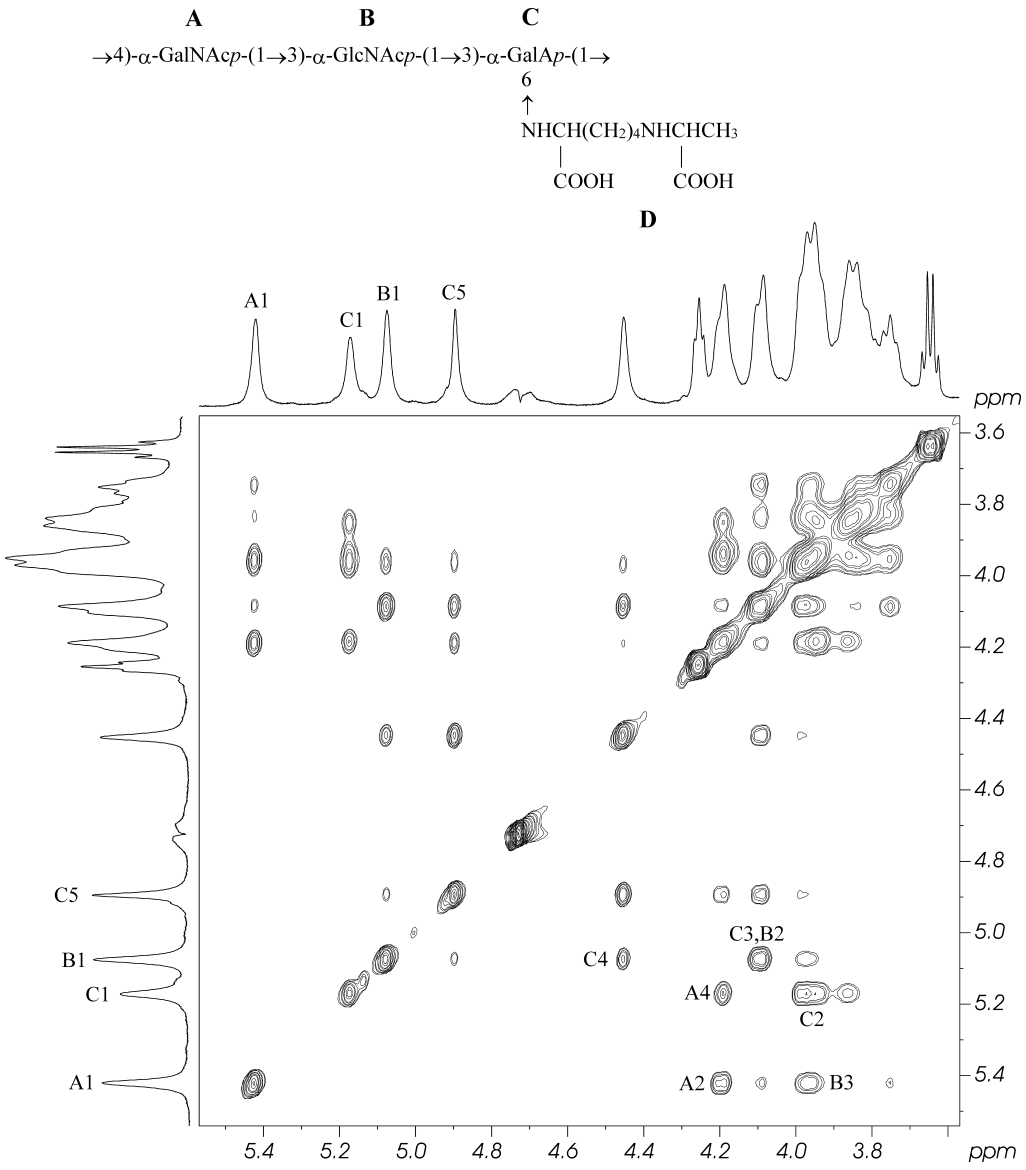


Fig. 3. Part of a NOESY spectrum of the O-specific polysaccharide of *P. rustigianii* O14. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to protons of sugar residues denoted by letters as shown in the structure at the top.

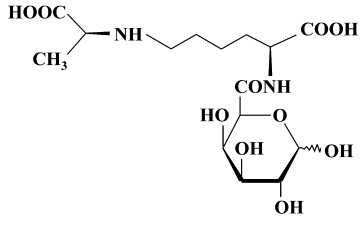
cally related bacteria *Proteus mirabilis* O13[14] and *Proteus myxofaciens*.[20]

Rabbit polyclonal antiserum against heat-killed cells of *P. rustigianii* O14 was tested in passive hemolysis test, enzyme-immunosorbent assay (EIA) and Western blot with the LPS of various *Providencia* and *Proteus* strains, including those containing N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine. Strong cross-reactions were observed with the LPS of *P. mirabilis* O13 and *P. myxofaciens* and significantly weaker cross-reactions with *P. alcalifaciens* O23 and two strains of *P. mirabilis* O3, S1959 and G1 (Table 3).

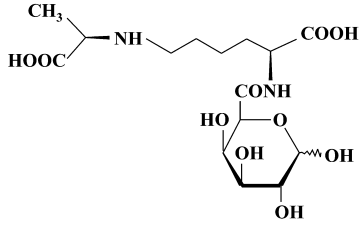
Western blot analysis showed that anti-*P. rustigianii* O14 serum recognized both slow and fast migrating bands of the homologous LPS corresponding to high- and low-molecular-mass LPS species with or without OPS chain, respectively (Fig. 6). The anti-*P. rustigianii* O14 serum reacted, to a different extent, also with high-molecular-mass LPS species of all cross-reactive LPS, thus indicating that the cross-reactive epitope(s) resides on the OPS moiety.

The structures of the cross-reactive O-antigens have been established[10,14,20–22] (Fig. 5). From them, O-antigens of *P. rustigianii* O14 and *P. mirabilis* O13 contain amides of the same uronic acid, D-GalA, with 2*S*,8*S*-AlaLys and 2*S*,8*R*-AlaLys, respectively, which may form a common epitope. This conclusion was confirmed by inhibition of the reaction in EIA in the homologous system anti-*P. rustigiani* O14 serum/*P. rustigiani* O14 LPS (Table 4). The LPS and OPS of

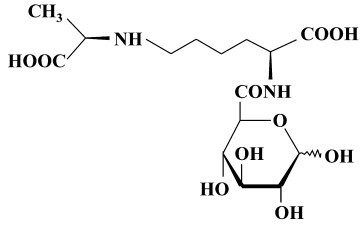
both *P. rustigianii* O14 and *P. mirabilis* O13 were strong inhibitors. Destruction of AlaLys by deamination of the OPS of *P. rustigianii* O14 and *P. mirabilis* O13 with nitrous acid significantly reduced or abolished the inhibiting activity, respectively. A similar loss of the serological reactivity was observed when the deaminated OPS of *P. mirabilis* O13 was tested with anti-*P. mirabilis* O13 serum.[23] These data demonstrate the importance of AlaLys for in the immunospecificity *P. rustigianii* O14 and the cross-reactivity with *P. mirabilis* O13. Remarkably, the absolute configuration of the 1-carboxyethyl group does not seem to play a significant role in antibody recognition.



N-(D-galacturonoyl)-*N*-[(*S*)-1-carboxyethyl)-L-lysine
Providencia rustigianii O14



N-(D-galacturonoyl)-*N*-[(*R*)-1-carboxyethyl)-L-lysine
Proteus mirabilis O13



N-(D-glucuronoyl)-*N*-[(*R*)-1-carboxyethyl)-L-lysine
Providencia alcalifaciens O23
Proteus myxofaciens

Fig. 4. Amides of uronic acids with N^{ε}-(1-carboxyethyl)lysine found in bacterial O-antigens. **2**, D-galacturonic acid with N^{α}-(D-galacturonoyl)-N^{ε}-[(*S*)-1-carboxyethyl)-L-lysine from *P. rustigianii* O14 (this work); N^{α}-(D-galacturonoyl)-N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine (**3**) from *P. mirabilis* O13,[14] and N^{α}-(D-glucuronoyl)-N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine (**4**) from *P. alcalifaciens* O23[10] and *P. myxofaciens*.[18]

From the LPS containing an amide of D-GlcA with 2*S*,8*R*-AlaLys, that of *P. myxofaciens* was a moderate inhibitor, whereas the LPS of *P. alcalifaciens* O23 inhibited the reaction insignificantly (Table 4). This distinction may be accounted for by a different accessibility of the AlaLys-associated epitope to antibodies, which is defined by three-dimensional structures of the O-antigens. Serological studies with O-antisera against *P. myxofaciens* and *P. alcalifaciens* O23, which demonstrate the importance of the amide of D-GlcA with 2*S*,8*R*-AlaLys for manifesting the immunospecificity of these bacteria, will be published elsewhere.

The O-antigens of *P. mirabillis* O3a,3b (S1959) and O3a,3c (G1) contain an amide of D-GalA with L-lysine (Fig. 5), which, most likely, is responsible for a weak cross-reactivity with anti-*P. rustigianii* O14 serum (Table 3, Fig. 6). N^{α}-(D-galacturonoyl)-L-lysine was demonstrated to play a role as an antigenic determinant in *P. mirabilis* O3 LPS.[22,24] The data obtained further confirmed that uronic acids and their amides with amino acids play an important role in the serological specificity of *Providencia* and *Proteus* O-antigens.[25]

The absence of inhibition with α-GalA-L-Lys-PAA (Table 4) suggested the importance of substitution of the lysine moiety with the 1-carboxyethyl group for manifesting the *Providencia* O14 specificity.

1. Experimental

1.1. Bacterial strain and growth

P. rustigianii O14:K1:H12 strain 1588 came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). The bacteria were cultivated under aerobic conditions in nutrient broth supplemented with 1% glucose. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilised.

1.2. Isolation and degradations of the LPS and the polysaccharide

LPS was isolated from bacterial cells by phenol–water extraction[11] and purified by treatment with cold aq 50% CCl_3CO_2H; the aqueous layer was dialysed and freeze-dried. The alkali-treated LPSs were prepared by saponification of the LPS with 0.25 M NaOMe in abs MeOH at 37 °C for 15 h.

A high-molecular-mass OPS was prepared by degradation of the LPS with aq 2% HOAc at 100 °C for 6 h followed by GPC of the water-soluble portion on a column (60 × 3.0 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring the elution using a Knauer differential refractometer

Providencia rustigianii O14

→4)-α-D-Gal*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→3)-α-D-Gal*p*A6(*2S,8S*-AlaLys)-(1→

Proteus mirabilis O13

α-D-Gal*p*A6(*2S,8R*-AlaLys)
1
↓
4
→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→

Proteus myxofaciens

→6)-β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A6(*2S,8R*-AlaLys)-(1→6)-α-D-Gal*p*NAc-(1→

Providencia alcalifaciens O23

→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*(1→3)-β-D-GalNAc*p*(1→4)-β-D-GlcA*p*6(*2S,8R*-AlaLys)-(1→

Proteus mirabilis S1959 (O3)

α-D-Gal*p*A6(L-Lys) α-D-Glc*p*
1 1
↓ ↓
4 2
→3)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*A-(1→

Proteus mirabilis G1 (O3)

α-D-Gal*p*A6(L-Lys)
1
↓
4
→3)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*A-(1→

Fig. 5. Structures of the O-specific polysaccharides of the serologically cross-reactive LPS.

(Germany). The yield of the OPS was 14.3% of the LPS mass. Deamination of the OPS was performed with 5% $NaNO_2$ in aq 20% HOAc at ambient temperature for 1 h, and the modified polysaccharide was isolated by gel chromatography on Sephadex G50 or TSK HW-40 (S).

1.3. Sugar analysis and isolation of amino acid

The OPS was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were converted into the alditol acetates[26] and analysed by GLC using a Hewlett-Packard 5880 instrument with a DB-5 capillary column and a temperature gradient of 160 (1 min) to 250 °C at 3 °C/min. The absolute configurations of the amino sugars were determined by GLC of the acetylated glycosides with (+)-2-octanol[12,13] under the same chromatographic conditions as above.

The OPS (12 mg) was hydrolysed with 2 M CF_3CO_2H (1 mL) at 120 °C for 2 h, acid was evaporated in vacuum and the products fractionated on a column (80 × 1.6 cm) of TSK HW-40 in water to give N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine in a yield 20% of the OPS mass.

1.4. Methylation analysis

Methylation was performed as described.[27] After hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h), the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated and analyzed by GLC–MS on a Hewlett-Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column and combined with

Table 3
Serological reactivity of anti-*P. rustigianii* O14 serum with the LPS of *Proteus* and *Providencia* (reciprocal titer)

Antigen from	PHT	EIA
P. rustigianii O14	12,800	64,000
P. mirabilis O13	12,800	32,000
P. myxofaciens	12,800	64,000
P. alcalifaciens O23	1600	4000
P. mirabilis O3a,3b (S1959)	3200	2000
P. mirabilis O3a,3c (G1)	1600	4000

Alkali-treated LPS and LPS were used in passive hemolysis test (PHT) and EIA, respectively.

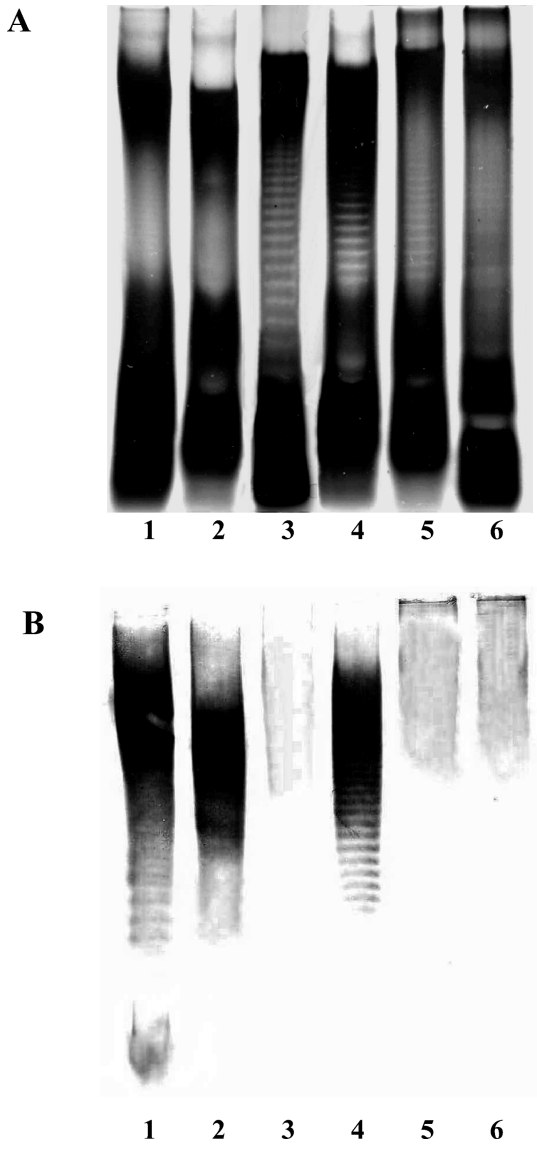


Fig. 6. Sodium deoxycholate polyacrylamide gel electrophoresis (A) and Western blot with anti-*P. rustigianii* O14 serum (B) of the LPS of *P. rustigianii* O14 (lane 1), *P. mirabilis* O13 (lane 2), *P. alcalifaciens* O23 (lane 3), *P. myxofaciens* (lane 4), *P. mirabilis* O3 (lane 5), *P. mirabilis* G1 (lane 6).

Table 4
Inhibition of the reaction in EIA of anti-*P. rustigianii* O14 serum with the homologous LPS

Inhibitor	Inhibitory dose (ng)
P. rustigianii O14 LPS	1.2
P. rustigianii O14 OPS	4.9
P. rustigianii O14 OPS-DA	1250
P. mirabilis O13 LPS	9.8
P. mirabilis O13 OPS	9.8
P. mirabilis O13 OPS-DA	>5000
P. myxofaciens LPS	156.1
P. alcalifaciens O23 LPS	5000
α-GalA-L-Lys-PAA	>50,000

OPS-DA, deaminated OPS; PAA, polyacrylamide.

a NERMAG R10-10L mass spectrometer, using a temperature gradient of 160 (1 min) to 250 °C at 3 °C/min.

1.5. NMR spectroscopy

Samples were adjust to pH 6 with pregnant solution of NaOD in D_2O, deuterium-exchanged by freeze-drying three times from D_2O, and then examined in a solution of 99.96% D_2O. Spectra were recorded using a Bruker DRX-500 spectrometer at 25° (1D ^{13}C NMR) or 57 °C (1D ^{1}H NMR and 2D NMR spectra). A mixing time of 200 ms was used in 2D TOCSY and NOESY experiments, respectively. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45).

1.6. Serological techniques

Rabbit polyclonal anti-*P. rustigianii* O14 serum was obtained by immunization of New Zealand white rabbits with heat killed bacteria as described.[28] Passive hemolysis test with alkali-treated LPS and EIA with LPS as antigen, inhibition of the reaction in EIA, sodium deoxycholate polyacrylamide gel electrophoresis and Western blot were performed as described previously.[29]

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