Available online at www.sciencedirect.com
SCIENCE DIRECT®
CARBOHYDRATE RESEARCH
ELSEVIER
Carbohydrate Research 338 (2003) 1425–1430
www.elsevier.com/locate/carres

Note

# Structure of the O-polysaccharide of *Providencia alcalifaciens* O21 containing 3-formamido-3,6-dideoxy-D-galactose

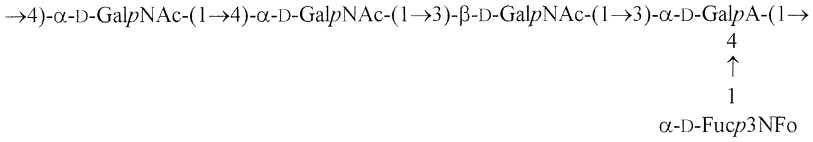
Nina A. Kocharova,[a] Agnieszka Maszewska,[b] George V. Zatonsky,[a] Olga V. Bystrova,[a] Andrzej Ziolkowski,[b] Agnieszka Torzewska,[b] Aleksander S. Shashkov,[a] Yuriy A. Knirel,[a] Antoni Rozalski[b,*]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, 119991 Moscow, GSP-1, Russia*
[b] *Department of Immunobiology of Bacteria, Institute of Microbiology and Immunology, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland*

Received 31 January 2003; accepted 11 April 2003

**Abstract**

The O-polysaccharide (O-antigen) of *Providencia alcalifaciens* O21 was obtained by mild acid degradation of the lipopolysaccharide and studied by chemical methods and NMR spectroscopy. It was found that the polysaccharide is built up of branched pentasaccharide repeating units with a terminal residue of 3-formamido-3,6-dideoxy-D-galactose (D-Fuc3NFo) and has the following structure:




Anti-*P. alcalifaciens* O21 serum cross-reacted with the O-antigen of *Proteus vulgaris* O47, which contains a GalNAc trisaccharide similar to that present in the *P. alcalifaciens* O21 O-polysaccharide.


*Keywords:* Lipopolysaccharide; O-Antigen; Bacterial polysaccharide structure; N-Formyl; *Providencia alcalifaciens*

Gram-negative bacteria of the genus *Providencia* are facultative pathogens, which can cause intestinal and urinary tract infections. Currently, the genus is subdivided into five species: *P. alcalifaciens*, *P. rustigianii*, *P. heimbache*, *P. rettgerii* and *P. stuartii*. Being a component of the normal intestinal flora, in favourable conditions bacteria *P. alcalifaciens* can cause infections, particularly, traveler's diarrhea.[1] The serological O-specificity of *Providencia* is defined by the structure of the O-specific polysaccharide chain (O-antigen) of the outer membrane lipopolysaccharide (LPS), which is also considered as a virulence factor of these bacteria. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 62 O-serogroups.[2] Recent structural studies of the O-polysaccharides[3–7] aim at establishing of the molecular basis for classification of *Providencia* strains. At present, structures of the O-polysaccharides of *P. alcalifaciens* O5,[3] O7,[4] O14[5], O16[6] and O23[7] have been established. Now we report on the structure of the O-polysaccharide of *P. alcalifaciens* O21.

The polysaccharide was obtained by mild acid degradation of the LPS, isolated from bacterial cells by the phenol–water procedure,[8] and purified by GPC on Sephadex G-50. Sugar analysis using GLC–MS of the acetylated alditols revealed 3-amino-3,6-dideoxygalactose (Fuc3N) and GalN in the ratio ~1:3. In addition, galacturonic acid (GalA) was identified by anion-exchange chromatography using a sugar analyser. The

* Corresponding author. Tel.: +48-42-6354464; fax: +48-42-6784932.
*E-mail address:* rozala@biol.uni.lodz.pl (A. Rozalski).

0008-6215/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00178-2

D configuration of the amino sugars was determined by GLC of the acetylated glycosides with (+)-2-octanol[9] and that of GalA by analysis of the $^{13}C$ NMR chemical shift data of the polysaccharide (see below). Methylation analysis revealed 3- and 4-substituted GalN residues and a terminal Fuc3N residue.

The $^{13}C$ NMR spectrum of the polysaccharide (Fig. 1) contained signals for five sugar residues, including signals for anomeric carbons at $\delta$ 95.5–104.7, one methyl group (C-6 of Fuc3N) at $\delta$ 17.0, four nitrogen-bearing carbons at $\delta$ 50.9 (2C), 51.6 and 52.1 (C-2 of GalN and C-3 of Fuc3N), other sugar carbons in the region $\delta$ 60.9–78.5, three *N*-acetyl groups at $\delta$ 23.4–23.6 (Me) and $\delta$ 175.8–176.2 (CO) and one *N*-formyl group at $\delta$ 165.7 and 168.8 (major and minor; Z and E isomers, respectively[15]). The $^{1}H$ NMR spectrum of the polysaccharide contained, inter alia, signals for five anomeric protons at $\delta$ 4.80–5.14, one methyl group (H-6 of Fuc3N) at $\delta$ 1.28, *N*-acetyl groups at $\delta$ 2.04 and one *N*-formyl group at $\delta$ 8.16 and 8.04 (Z and E isomers, respectively[10]).

To determine the position of the formyl group the polysaccharide was N-deformylated by mild acid treatment and than N-acetylated with trideuterioacetanhydride. Acid hydrolysis of the modified polysaccharide under the conditions that retained *N*-acetyl groups followed by GLC–MS analysis of the acetylated alditols derived revealed 3-trideuterioacetamido-3,6-dideoxygalactose. Hence, the formyl group is attached to Fuc3N and 3,6-dideoxy-3-formamidogalactose (Fuc3NFo) is present in the *P. alcalifaciens* O21 polysaccharide.

The $^{1}H$ and $^{13}C$ NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, NOESY, and H-detected $^{1}H$, $^{13}C$ HSQC experiments (Tables 1 and 2). The five monosaccharide spin systems were assigned based on the coupling constant values, which were estimated from the $^{1}H$, $^{1}H$ COSY and TOCSY spectra. In the TOCSY spectrum, there were cross-peaks for H-1 with H-2,3,4 of GalN (units **A**–**C**) and GalA (unit **D**) and H-2,3,4,5 of Fuc3N (unit **E**). The $J_{1,2}$ values showed that GalA, Fuc3N and two GalN residues (units **A** and **B**) are α-linked, whereas the third GalN residue (unit **C**) is β-linked.

The sequence of the monosaccharides in the repeating unit was determined by a NOESY experiment (Fig. 2), which showed the following interresidue cross-peaks between the anomeric protons and protons at the linkage carbons: α-GalNAc (**A**) H-1,α-GalNAc (**B**) H-4; α-GalNAc (**B**) H-1,β-GalNAc (**C**) H-3; β-GalNAc (**C**) H-1,α-GalA (**D**) H-3; α-GalA (**D**) H-1,α-GalNAc (**A**) H-4 and α-Fuc*p*3NFo (**E**) H-1,α-GalA (**D**) H-4 at $\delta$ 5.00/4.07, 5.10/3.80, 4.80/4.25, 5.14/4.18 and 4.97/4.65, respectively. The glycosylation pattern of the polysaccharide was confirmed by significant downfield displacements of the signals for C-4 of α-GalNAc (**A** and **B**), C-3 of β-GalNAc (**C**), and C-3 and C-4 of α-GalA (**D**) to $\delta$ 78.5, 78.0, 77.2, 78.0 and 76.8, respectively, as compared with their position in the corresponding nonsubstituted monosaccharides.[11,12]

The D configuration of the sugar residue in GalA was demonstrated by a relatively large glycosylation effect ($>7$ ppm) on C-1 of β-D-GalNAc, which is characteristic for the same absolute configurations of the monosaccharides in the β-D-Gal*p*NAc-(1→3)-D-GalA disaccharide fragment (an effect of $<5$ ppm would be observed in case of different absolute configurations of the monosaccharides[13]).

These data showed that the O-specific polysaccharide of *P. alcalifaciens* O21 has the following structure:

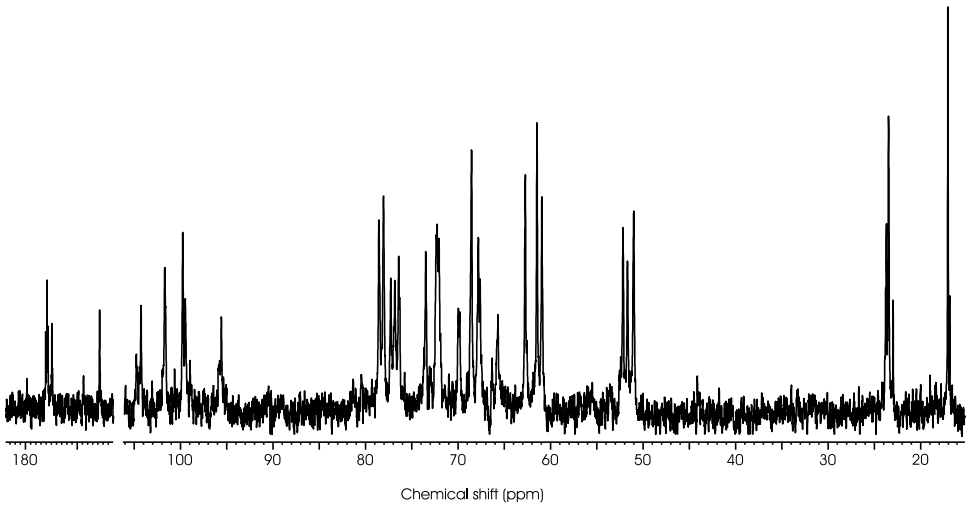
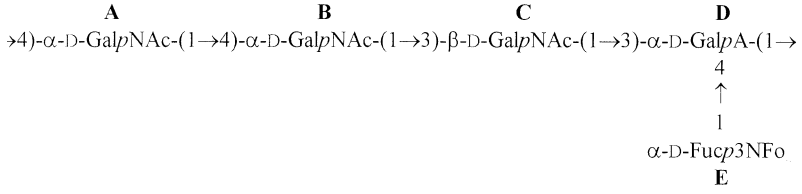



Fig. 1. 125-MHz $^{13}C$ NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O21.

Table 1
500-MHz $^{1}$H NMR chemical shifts of the O-polysaccharide of *P. alcalifaciens* O21 ($\delta$, ppm)

| Sugar residue | | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b |
|---|---|---|---|---|---|---|---|---|
| →4)-α-D-Gal*p*NAc-(1→ | (**A**) | 5.00 | 4.24 | 4.16 | 4.18 | 4.41 | 3.79 | 3.79 |
| →4)-α-D-Gal*p*NAc-(1→ | (**B**) | 5.10 | 4.30 | 3.91 | 4.07 | 3.91 | 3.72 | 3.72 |
| →3)-β-D-Gal*p*NAc-(1→ | (**C**) | 4.80 | 4.15 | 3.80 | 4.12 | 3.64 | 3.89 | 3.77 |
| →3,4)-α-D-Gal*p*A-(1→ | (**D**) | 5.14 | 4.15 | 4.25 | 4.65 | 4.85 | | |
| α-D-Fuc*p*3NFo-(1→ | (**E**) | 4.97 | 3.71 | 4.22 | 3.72 | 4.53 | 1.28 | |

The signal for NAc is at $\delta$ 2.04 and those for NFo are at $\delta$ 8.04 (minor) and 8.16 (major).




A peculiar feature of the polysaccharide is the presence of 3-formamido-3,6-dideoxy-D-galactose, which, to the best of our knowledge, has not been found hitherto in bacterial polysaccharides.

The LPS from *P. alcalifaciens* O21 and those from various *Providencia* and *Proteus* strains that have structurally similar O-polysaccharides (Fig. 3) were tested in passive hemolysis, enzyme-immunosorbent assay (EIA) and Western blot with rabbit polyclonal antiserum against heat-killed bacteria of *P. alcalifaciens* O21. A strong reaction was observed with the homologous LPS in all tests and a marked cross-reactivity with the LPS of *Proteus vulgaris* O45 and O47 in passive hemolysis test (Table 3). In EIA, all heterologous LPS tested showed a negligible cross-reactivity (Table 3). The differences in the reactivity of *P. alcalifaciens* O21 serum in passive hemolysis and EIA could be accounted for by a different exposure of the carbohydrate epitope on the LPS dependent on the antigen carrier used in the serological assays (sheep red blood cells and plastic surface, respectively).

In Western blot (Fig. 4), anti-*P. alcalifaciens* O21 serum recognized both slow and fast migrating bands of the homologous LPS, which correspond to high- and low-molecular-mass LPS species with and without O-polysaccharide chain, respectively. It reacted also with high molecular-mass LPS species of *P. vulgaris* O47, thus indicating that a cross-reactive epitope(s) resides on the O-polysaccharide part. Comparison of the O-polysaccharide structure of *P. alcalifaciens* O21 established in this work with that of *P. vulgaris* O47 determined by us earlier[14] (Fig. 3) revealed the presence in the main chain of each polysaccharide of a trisaccharide fragment built up of three GalNAc residues, which may be responsible for the cross-reactivity. However, the linkages in two trisaccharides are different and, correspondingly, the cross-reactivity is only weak. Most antibodies in anti-*P. alcalifaciens* O21 serum recognize

Table 2
125-MHz $^{13}$C NMR chemical shifts of the O-polysaccharide of *P. alcalifaciens* O21 ($\delta$, ppm)

| | | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|---|
| →4)-α-D-Gal*p*NAc-(1→ | (**A**) | 99.7 | 51.6 | 69.9 | 78.5 | 72.3 | 60.9 |
| →4)-α-D-Gal*p*NAc-(1→ | (**B**) | 95.5 | 50.9 | 68.5 | 78.0 | 73.4 | 61.4 |
| →3)-β-D-Gal*p*NAc-(1→ | (**C**) | 104.7 | 52.1 | 77.2 | 65.6 | 76.4 | 62.7 |
| →3,4)-α-D-Gal*p*A-(1→ | (**D**) | 101.6 | 67.8 | 78.0 | 76.8 | 72.2 | 175.0 |
| α-D-Fuc*p*3NFo-(1→ | (**E**) | 99.4 | 67.5 | 50.9 | 72.0 | 68.5 | 17.0 |

Signals for NAc are at $\delta$ 23.4–23.6 (Me) and 175.8, 175.9 and 176.2 (all CO); for NFo at $\delta$ 165.7 (major) and 168.8 (minor).

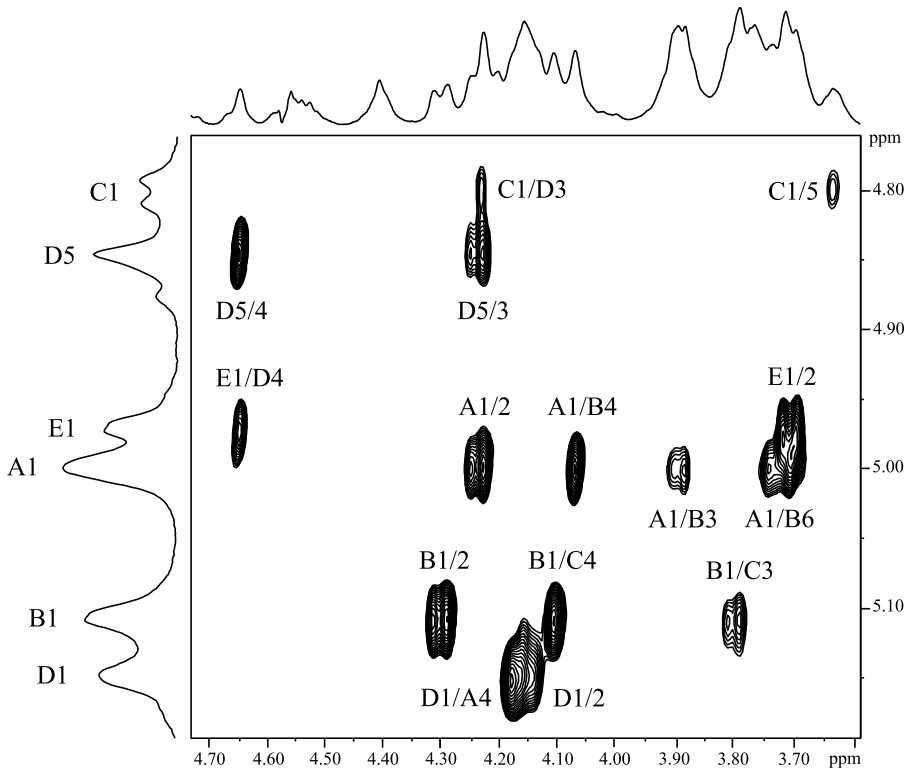



Fig. 2. Part of a 500-MHz NOESY spectrum of the O-polysaccharide of *P. alcalifaciens* O21. The corresponding parts of the $^1$H NMR spectrum are shown along the axes.

a unique epitope on the homologous O-polysaccharide, which, most likely, is associated with the lateral Fuc3NFo residue.

In contrast, only low-molecular-mass LPS species of *P. vulgaris* O45 cross-reacted weakly with anti-*P. alcalifaciens* O21 serum in Western blot (Fig. 4). This finding indicated sharing of an epitope(s) on the LPS core of *P. alcalifaciens* O21 and *P. vulgaris* O45, whose structure remains to be determined. The unique structure of the O-antigen of *P. alcalifaciens* O21 and the serological data are in agreement with classification of this bacterium in a separate *Providencia* serogroup.

## 1. Experimental

### 1.1. Bacterial strain and isolation of the LPS

*Providencia alcalifaciens* O21:H23, strain 5241/50, from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was 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.

The LPS was isolated from bacterial cells by phenol–water extraction[8] and purified by treatment with cold aq 50% $CCl_3CO_2H$; the aqueous layer was dialysed and freeze-dried.

### 1.2. Isolation and modification of the O-specific polysaccharide

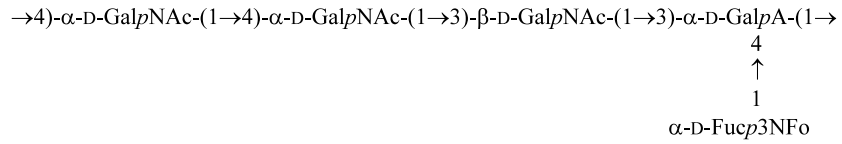
The O-specific polysaccharide was obtained by mild acid hydrolysis of the LPS with 0.1 M AcONa buffer pH 4.2 for 12 h followed by GPC of the water-soluble portion on a column (60 × 2.5 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring using a Knauer differential refractometer (Germany). The yield of the polysaccharide was 10.6% of the LPS weight.

The polysaccharide was treated with 0.05 M HCl (100 °C, 1 h) and, after lyophilization, the product was N-acetylated with $(CD_3CO)_2O$ as described.[16]

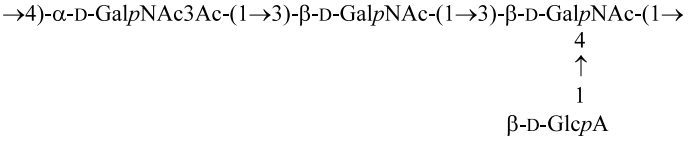
### 1.3. Sugar analysis

The polysaccharide was hydrolysed with 2 M $CF_3CO_2H$ (120 °C, 2 h), and the modified polysaccharide with 4 M HCl (65 °C, 4 h). Amino sugars were converted into the alditol acetates[17] and analysed by GLC–MS on a Hewlett-Packard 5880 instrument with a DB-5 capillary column and a NERMAG R10-10L mass spectrometer using a temperature gradient of 160 °C (1 min) to 290 °C at 3 °C/min. Uronic acids were analysed on a Biotronik

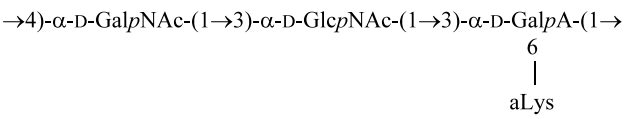
*Providencia alcalifaciens* O21 (this work)



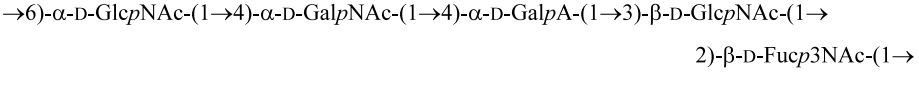

*Proteus vulgaris* O47[14]




*Providencia rustigiani* O14[5]




*Proteus vulgaris* O45[14]




*Proteus mirabilis* O29[15]

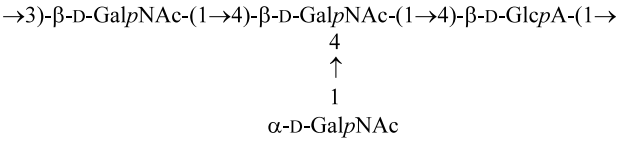



Fig. 3. Structures of the O-polysaccharides of the *Providencia* and *Proteus* LPS used in serological studies. Fuc3NFo stands for 3,6-dideoxy-3-formamidogalactose and aLys for $N^{\varepsilon}$-[(*S*)-1-carboxyethyl]-L-lysine ('alaninolysine').

LC-2000 sugar analyzer (Germany) on a column of Dionex A × 8 anion-exchange resin at 70 °C using 0.04 M sodium phosphate buffer pH 2.4. The absolute configurations of amino sugars were determined by GLC of the acetylated glycosides with (+)-2-octanol[9,18] under the same chromatographic conditions as above.

Table 3
Serological reactivity of anti-*P. rustigianii* O21 serum with the LPS of *Providencia* and *Proteus* (reciprocal titer)

| Antigen form | Passive hemolysis | EIA |
|---|---|---|
| *P. alcalifaciens* O21 | 102,400 | 2,049,000 |
| *P. rustigianii* O14 | 400 | 8000 |
| *P. vulgaris* O45 | 6400 | 2000 |
| *P. vulgaris* O47 | 12,800 | 8000 |
| *P. mirabilis* O29 | 200 | 1000 |

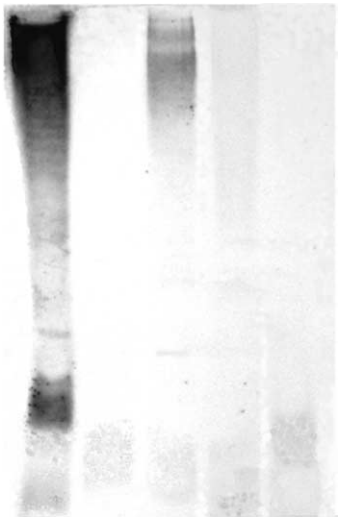
Alkali-treated LPS or LPS was used as antigen in passive hemolysis and EIA, respectively.

### 1.4. Methylation analysis

Methylation of the polysaccharide was performed as described.[19] 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 the same instrument as in sugar analysis using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min.

### 1.5. NMR spectroscopy

Polysaccharide samples were deuterium-exchanged by freeze-drying three times from 99.9% $D_2O$ and then



1 2 3 4 5

Fig. 4. Western blot of the LPS of *P. alcalifaciens* O21 (lane 1), *P. vulgaris* O45 (lane 2), *P. vulgaris* O4 (lane 3) *P. rustigianii* O14 (lane 4) and *Proteus mirabilis* O29 (lane5) with anti-*P. alcalifaciens* O21 serum.

examined in 99.96% $D_2O$. Spectra were recorded using a Bruker DRX-500 spectrometer using standard Bruker software. A mixing time of 150 and 200 ms was used in 2D TOCSY and NOESY experiments, respectively.

### 1.6. Serological techniques

Rabbit polyclonal anti-*P. rustigianii* O21 serum was obtained by immunization of New Zealand white rabbits with heat-killed bacteria as described[20] Passive hemolysis with alkali-treated LPS and EIA with LPS as antigen, DOC-PAGE and Western blot were performed as described previously.[21]

## Acknowledgements

This work was supported by grant 02-04-48118 of the Russian Foundation for Basic Research, and grant 3P05 037 22 of the Science Research Committee (KBN, Poland). We thank Mgr Marianna Wykrota for excellent technical assistance.

## References

1. O'Hara, C. M.; Brenner, F. W.; Miller, J. M. *Clin. Microbiol. Rev.* **2000**, *13*, 534–546.
2. Ewing, W. H. In *Identification of Enterobacteriaceae*; Edwards, P. R., Ed.; Elsevier: New York, 1986; pp 454–459.
3. Zatonsky, G. V.; Bystrova, O. V.; Kocharova, N. A.; Shashkov, A. S.; Knirel, Y. A.; Kholodkova, E. V.; Stanislavsky, E. S. *Biochemistry (Moscow)* **1999**, *64*, 628–633.
4. Bystrova, O. V.; Zatonsky, G. V.; Borisova, S. A.; Kocharova, N. A.; Shashkov, A. S.; Knirel, Y. A.; Kholodkova, E. V.; Stanislavsky, E. S. *Biochemistry (Moscow)* **2000**, *65*, 677–684.
5. Kocharova, N.A.; Zatonsky, G.V.; Torzewska, A.; Macieja, Z.; Bystrova, O.V.; Shashkov, A.S.; Knirel, Y.A.; Rozalski, A. *Carbohydr. Res.* **2003**, in press.
6. Kocharova, N. A.; Zatonsky, G. V.; Bystrova, O. V.; Ziolkowski, A.; Wykrota, M.; Shashkov, A. S.; Knirel, Y. A.; Rozalski, A. *Carbohydr. Res.* **2002**, *337*, 1667–1671.
7. Kocharova, N. A.; Vinogradov, E. V.; Borisova, S. A.; Shashkov, A. S.; Knirel, Y. A. *Carbohydr. Res.* **1998**, *309*, 131–133.
8. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–89.
9. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
10. Katzenellenbogen, E.; Romanowska, E.; Kocharova, N. A.; Shashkov, A. S.; Knirel, Y. A.; Kochetkov, N. K. *Carbohydr. Res.* **1995**, *273*, 187–195.
11. Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59–75.
12. Jansson, P.-E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *188*, 169–191.
13. Shashkov, A. S.; Lipkind, G. M.; Knirel, Y. A.; Kochetkov, N. K. *Magn. Reson. Chem.* **1988**, *26*, 735–747.
14. Różalski, A.; Torzewska, A.; Bartodziejska, B.; Babicka, D.; Kwil, I.; Perepelov, A. V.; Kondakova, A. N.; Senchenkova, S. N.; Knirel, Y. A.; Vinogradov, E. V. *Wiadomości Chemiczne* **2002**, *56*, 585–604.
15. Perepelov, A. V.; Shashkov, A. S.; Senchenkova, S. N.; Knirel, Y. A.; Literacka, E.; Kaca, W. *Biochemistry (Moscow)* **2000**, *65*, 176–179.
16. Holst, O.; Broer, W.; Thomas-Oates, J. E.; Mamat, U; Brade, H. *Eur. J. Biochem.* **1993**, *214*, 703–710.
17. Sawardeker, J. S.; Sloneker, J. H.; Jeanes, A. *Anal. Chem.* **1965**, *37*, 1602–1603.
18. Shashkov, A. S.; Senchenkova, S. N.; Nazarenko, E. L.; Zubkov, V. A.; Gorshkova, N. M.; Knirel, Y. A.; Gorshkova, R. P. *Carbohydr. Res.* **1997**, *303*, 333–338.
19. Conrad, H. E. *Methods Carbohydr. Chem.* **1972**, *6*, 361–364.
20. Bartodziejska, B.; Shashkov, A. S.; Babicka, D.; Grachev, A. A.; Torzewska, A.; Paramonov, N. A.; Chernyak, A. Y.; Rozalski, A.; Knirel, Y. A. *Eur. J. Biochem.* **1998**, *256*, 488–493.
21. Torzewska, A.; Kondakova, A. N.; Perepelov, A. V.; Senchenkova, S. N.; Shashkov, A. S.; Rozalski, A.; Knirel, Y. A. *FEMS Immunol. Med. Microbiol.* **2001**, *31*, 227–234.