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Proteus mirabilis RMS 203 as a new representative of the O13 *Proteus* serogroup*

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The unique feature of some *Proteus* O-polysaccharides is occurrence of an amide of galacturonic acid with *N*ε-[(*S/R*)-1-Carboxyethyl]-L-lysine, GalA6(*2S,8S/R*-AlaLys). The results of the serological studies presented here, with reference to known O-antigens structures suggest that GalA6(*2S,8S/R*-AlaLys) or *2S,8R*-AlaLys contribute to cross-reactions of O13 *Proteus* antisera, and *Proteeae* LPSs. It was also revealed that the *Proteus mirabilis* RMS 203 strain can be classified into the O13 serogroup, represented so far by two strains: *Proteus mirabilis* 26/57 and *Proteus vulgaris* 8344. The O13 LPS is a serologically important antigen with a fragment common to LPSs of different species in the *Proteeae* tribe.

Key words: cross-reaction, lipopolysaccharide, O-polysaccharide, O serogroup, *Proteus*

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INTRODUCTION

Bacteria belonging to the genus *Proteus* are Gram-negative, rod-shaped, peritrichously flagellated microorganisms, which together with *Providencia* and *Morganella* strains constitute the tribe *Proteeae* (Penner, 1992; O'Hara *et al*., 2000). They are opportunistic pathogens causing various kinds of infections with the domination of urinary tract infections (UTIs) (Coker *et al*., 2000). The hallmark of UTIs caused by *Proteus* spp. rods is their ascending character and occurrence of urinary stones, located most frequently in the kidney. Bacteria closed in the porous structure of the calculi are often the reason for the failure of antibiotic therapy and recurrence of the illness (Ambruster & Mobley, 2012; Torzewska *et al*., 2014). Among all species in the mentioned genus (*P. mirabilis*, *P. vulgaris, P. penneri*, *P. hauseri*, *P. myxofaciens* as well as three unnamed genomospecies: 4, 5 and 6), *P. mirabilis* is the most commonly isolated in all types of infections (Armbruster & Mobley, 2012).

One of the virulence factors of *Proteus* spp. bacilli is their outer membrane constituent — lipopolysaccharide (LPS) (Różalski *et al*., 1997; 2012). Its most external part: the O-polysaccharide (OPS), is a highly immunogenic O-antigen of these bacteria, and the varieties in its chemical composition are the basis for O-serotyping of *Proteus* spp. strains. Currently, there are 80 different O-serogroups described in this genus and some of them are divided into two or even more subgroups (Sidorczyk *et al*., 2002; Kaca *et al*., 2011; Knirel *et al*., 2011; Siwińska *et al*., 2015). Strains classified into some of these serogroups (for example *Proteus* O13, but also O3, O10 and O26) had previously been reported to be the most frequent among *Proteus* spp. clinical isolates (Larsson, 1984).

Proteus bacilli LPSs are known to be acidic due to the occurrence of characteristic compounds like: D-glucuronic or D-galacturonic acids (GlcA and GalA, respectively) as well as: (*R*) or (*S*) lactic acid residues linked to the most common sugars: D-glucosamine or 2-acetamido-2-deoxy-D-glucose (GlcNAc). Other acidic constituents, like pyruvic acid, have been also identified. It is worth emphasizing that in some *Proteus* spp. OPSs, hexuronic acids, GalA and GlcA, can be additionally amidated with amino acids, like L-lysine, L-serine or L-alanine, however the L-lysine residue is the most frequent (Shashkov *et al*., 1997; Knirel *et al*., 2011). The unique feature of some *Proteus* species OPSs is the occurrence of *N*ε-[(*S/R*)-1-Carboxyethyl]-L-lysine, a component which was identified and described for the first time in the O13 *Proteus* OPS (Shashkov *et al*., 1997; Knirel *et al*., 2011). This unusual amino acid is also identified in polysaccharides of other representatives of the *Proteeae* tribe, for example in the OPS of *Providencia rustigianii* O14, however, in the last mentioned one it appears as an amide of GalA with *N*ε-[(*S*)-1-Carboxyethyl]-L-lysine (Kocharova *et al*., 2003).

As mentioned above, *Proteus* spp. bacilli O-polysaccharides are immunogenic and major and minor epitopes have been established for many of them (Zych *et al*., 2005; 2007). Here, we present the serospecificity of the *P. mirabilis* RMS 203 LPS, predicted to be the third member of the O13 serogroup, and describe the role of the *N*ε-[(*S/R*)-1-Carboxyethyl]-L-lysine as a cross-reactive epitope located in the O-antigens of *Proteus* spp. and *Providencia* spp. LPSs.

MATERIALS AND METHODS

Bacterial strains. *P. mirabilis* RMS 203 (SANK 70461) was provided by Professor K. Kodama, Clinic of Culture Collection, Tsukuba Research Laboratories, Sankyo Co., Ibaraki (Japan). The *P. mirabilis* 26/57 and 34/57 strains were from the Czech National Collection of Type Cultures, Institute of Epidemiology and Microbiology, Prague (Czech Republic). The *P. vulgaris* 8344 strain was kindly provided by Dr. M. Valvano, Department of Mi-

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Abbreviations: CFU, colony forming unit; ELISA, enzyme-linked immunosorbent assay; GalNAc, 2-acetamido-2-deoxy-D-galactose; Gal, galactose; GalA6(*2S,8S*-AlaLys), *N*ε-[(*S*)-1-Carboxyethyl]-*N*-(D-galacturonoyl)-L-lysine; GlcA6(*2S,8R*-AlaLys), amide of glucuronic acid with *N*ε-[(*R*)-1-Carboxyethyl]-L-lysine; GlcNAc, 2-acetamido-2-deoxy-D-glucose; LPS, lipopolysaccharide; OPS, O-polysaccharide; PBS, phosphate-buffered saline; UTIs, urinary tract infections.

crobiology and Immunology, University of Western Ontario (Canada). The *P. myxofaciens* strain 18769-CCUG (ATCC 19692) was kindly provided by Dr. E. Falsen, the Culture Collection, University of Goeteborg (Sweden). The tested *Proteus* spp. strains belong to the collection of the Department of General Microbiology, University of Łódź, Poland. The *P. rustigianii* O14:K1:H12 strain 1588 came from the collection of the Department of Immunobiology of Bacteria, University of Łódź (Poland).

Lipopolysaccharide. The *P. mirabilis* RMS 203 LPS was extracted from dried bacterial cells by the phenol-water procedure according to the method by Westphal and purified with aqueous 50% trichloroacetic acid (Westphal & Jann, 1965). The other tested *Proteus* spp. LPSs, obtained and purified by the above-mentioned method, came from the collection of the Department of General Microbiology, University of Łódź. The *P. rustigianii* O14 LPS was obtained from the Department of Immunobiology of Bacteria, University of Łódź.

Rabbit antisera. Polyclonal O-antiserum against *P. mirabilis* RMS 203 was obtained by the immunization of a rabbit with heat-inactivated bacteria. The immunization scheme included three injections at intervals of five days. The bacterial suspension (1.5×10^{10} CFU/ml) was applied in doses of 0.25, 0.5 and 1.0 ml per rabbit. Seven days after the last injection, the rabbits were bled. Sera against *P. mirabilis* 26/57 and *P. vulgaris* 8344, obtained by the same method as the *P. mirabilis* RMS 203 antiserum, came from the collection of the Department of General Microbiology, University of Łódź.

Serological assays. Purified LPS samples were tested with rabbit antisera in an enzyme-linked immunosorbent assay (ELISA) and the Western blot procedure after sodium dodecyl sulfate polyacrylamide gel electrophoresis with unadsorbed antisera and/or antisera adsorbed with the bacterial mass of tested strains. In the adsorption procedure, a wet mass of bacterial cells (washed previously in phosphate-buffered saline — PBS) was suspended in serum diluted 1:50 in PBS. After 30 minutes of incubation on ice, the bacterial cells were removed by centrifugation. The procedure was performed three times for each serum tested (Drzewiecka *et al.*, 2008). Other assays were performed as previously described (Sidorczyk *et al.*, 2002).

RESULTS AND DISCUSSION

To date, the O13 *Proteus* serogroup was represented by two strains: *P. mirabilis* 26/57 and *P. vulgaris* 8344 (Knirel *et al.*, 2011).

In this work, the serospecificity of the *P. mirabilis* RMS 203 LPS, predicted to be a third member of the O13 serogroup, is presented. What is the most important for the serological classification, these studies were performed by use of three sera specific to: *P. mirabilis* 26/57, RMS 203 and *P. vulgaris* 8344 strains and their LPSs, which allowed showing the whole spectrum of the cross-reactions. Moreover, due to the structural similarities of *P. mirabilis* 26/57 and *P. vulgaris* 8344 OPSs to the O-antigens of *P. myxofaciens* (O60) and *Providencia rustigianii* (O14) LPSs (Kocharova *et al.*, 2003; Knirel *et al.*, 2011), the latter two were also included in these studies to check how far these structural similarities are reflected in the LPSs cross-reactivity with the sera tested.

In the first stage of this study, the LPSs were examined in ELISA using the antisera tested (after or without their adsorption with a wet mass of bacterial cells of the strains used in the studies). The reactivity titer equal to 1:1,000 was regarded as significant. Using the adsorbed sera allowed showing the differences and similarities in serological specificities of the tested LPSs.

To estimate which part of LPS molecules contributed to the reactions, all tested systems were examined in Western blot. The results are presented in Table 1 and Fig. 1.

The *P. mirabilis* 26/57, RMS 203 and *P. vulgaris* 8344 LPSs formed a group of antigens reacting similarly in ELISA (the same reactivity titers with each unadsorbed serum) (Table 1, control) and in a Western blot (similar banding patterns observed at the level of high-molecular-mass species restricted to the LPS molecules with OPS chains) (Fig. 1). Using an adsorption procedure allowed abolishing the most reactions of the *P. mirabilis* 26/57, RMS 203 and *P. vulgaris* 8344 LPSs with the tested antisera (Table 1). Slight differences in these three LPSs specificity were noted in the following reactions:

- *P. mirabilis* 26/57 antiserum (adsorbed by *P. vulgaris* 8344 bacterial mass) with *P. mirabilis* RMS 203 LPS,
- *P. mirabilis* 26/57 antiserum (adsorbed by *P. mirabilis* RMS 203 bacterial mass) with *P. mirabilis* 26/57 LPS and that antiserum with *P. vulgaris* 8344 LPS,
- *P. mirabilis* RMS 203 antiserum (adsorbed by *P. mirabilis* 26/57 bacterial mass or by *P. vulgaris* 8344 bacterial mass) with *P. mirabilis* RMS 203 LPS (Table 1).

In the Western blot technique the differences in serospecificity of the core oligosaccharides of the *P. mirabilis* RMS 203 LPS and two representatives of the O13 serogroup were revealed by a lack of the following reactions: 1) *P. mirabilis* 26/57 antiserum with low-molecular-mass species of the *P. mirabilis* RMS 203 LPS; 2) *P. mirabilis* RMS 203 antiserum with low-molecular-mass species of the *P. mirabilis* 26/57 and *P. vulgaris* 8344 LPSs (Fig. 1a, c). Interestingly, the differences in the fast migrating bands of the *P. mirabilis* 26/57 and RMS 203 LPSs were not seen in a Western blot with the serum specific to the *P. vulgaris* 8344 strain (Fig. 1b), in which the bands of both LPSs were almost identical. Huge similarities in these LPSs serological activities were also confirmed in ELISA where the reactions of *P. vulgaris* 8344 antiserum (adsorbed with bacterial mass of the *P. mirabilis* 26/57 or with bacterial mass of *P. mirabilis* RMS 203) with their LPSs, were completely abolished (Table 1). A similar situation, where serological similarities of the core regions of two LPSs, *P. penneri* 40 and 62, had been previously observed in Western blot only for one serum and not for the other (Palusiak *et al.*, 2013). In both cases, such discrepancies may be explained by a different composition of core-specific antibodies in the tested sera: specific to the *P. vulgaris* 8344 strain (a higher level of core-specific antibodies compared to the *P. mirabilis* RMS 203 and 26/57 antisera) (this study) and to *P. penneri* 40 strain (a higher level of core-specific antibodies compared to the *P. penneri* 62 antiserum) (Palusiak *et al.*, 2013).

To confirm the similarities and differences appearing in the serological specificities of the *P. mirabilis* 26/57, *P. vulgaris* 8344 and *P. mirabilis* RMS 203 LPSs core regions, the fourth LPS, *P. mirabilis* 34/57 (with the core region specificity similar to that of the *P. mirabilis* RMS 203 LPS — data not shown) was additionally checked by ELISA and Western blotting with the *P. mirabilis* RMS 203 antiserum (Table 1, Fig. 1c). The antibodies in the tested serum bound only to fast migrating bands (restricted to core-lipid A molecules) of *P. mirabilis* 34/57 and the reaction was similar to, but stronger than, the one obtained for the homologous LPS. The differences in the reactions strength may have resulted from the lower number of *P. mirabilis* 34/57 LPS molecules with-

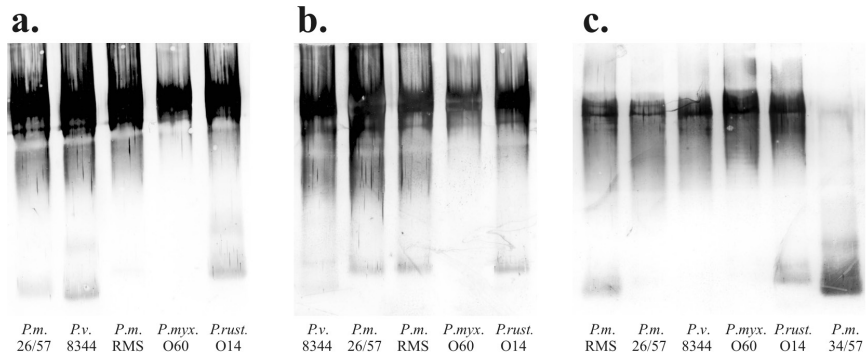


Figure 1. Western blot of *Proteus* spp. and *Providencia* spp. LPSs with sera specific to the strains: *P. mirabilis* 26/57 (O13) (a); *P. vulgaris* 8344 (O13) (b); *P. mirabilis* RMS 203 (c).

out the OPS chains compared to the *P. mirabilis* RMS 203 LPS, thus the access of antibodies to the *P. mirabilis* 34/57 LPS core region was better than in the case of the homologous LPS. A moderate high level of antibodies remaining in the *P. mirabilis* RMS 203 antiserum, after its adsorption with *P. mirabilis* 34/57 LPS, is probably connected with their reactivity with the OPS chains of the homologous LPS (Table 1). It is worth mentioning that the OPS part of the *P. mirabilis* 34/57 (O18) LPS was not recognized by the O13 antiserum. The slight smearing band observed in the Western blot technique at the level corresponding to the *P. mirabilis* 34/57 LPS moieties with OPS chains (Fig. 1c) disappeared after extending the path of LPS electrophoresis (data not shown).

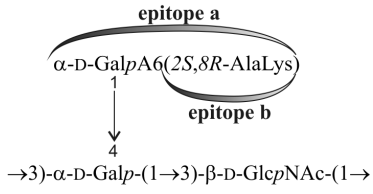
Another LPS of serological specificity very similar to that of *P. mirabilis* 26/57, RMS 203 and *P. vulgaris* 8344 LPSs appeared to be that of *P. rustigianii* O14 strain. It strongly reacted in a Western blot with each serum tested at the level of high-molecular mass species of its LPS and weakly at a level corresponding to low-molecular mass LPS species (Fig. 1). The significant decrease in the reactivity of each antiserum adsorbed by *P. rustigianii*

Table 1. The reactivity of *Proteus* spp. and *P. rustigianii* LPSs with the *P. mrabilis* 26/57, RMS 203 and *P. vulgaris* 8344 antisera in ELISA[a,b,c]

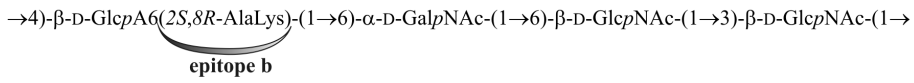
Antiserum/ adsorbed with:	Reciprocal titer of antiserum with the LPS from strains					
	P. mirabilis		*P. vulgaris*	*P. rustigianii*	*P. myxofaciens*	*P. mirabilis*
	26/57	RMS	8344	O14	O60	34/57
***P. mirabilis* 26/57**						
[a]Control	[b]**64.000**	64.000	64.000	32.000	32.000	[c]-
P. mirabilis 26/57	**<1.000**	<1.000	<1.000	<1.000	<1.000	-
P. mirabilis RMS 203	**1.000**	<1.000	2.000	<1.000	<1.000	-
P. vulgaris 8344	**<1.000**	1.000	<1.000	<1.000	<1.000	-
P. rustigianii	**1.000**	2.000	1.000	<1.000	<1.000	-
P. myxofaciens	**1.000**	2.000	4.000	1.000	<1.000	-
***P. mirabilis* RMS 203**						
[a]Control	128.000	[b]**128.000**	128.000	64.000	32.000	16.000
P. mirabilis RMS 203	<1.000	**<1.000**	<1.000	<1.000	<1.000	<1.000
P. mirabilis 26/57	<1.000	**1.000**	<1.000	<1.000	<1.000	[c]-
P. vulgaris 8344	<1.000	**1.000**	<1.000	<1.000	1.000	-
P. rustigianii	1.000	**2.000**	1.000	<1.000	<1.000	-
P. myxofaciens	2.000	**2.000**	2.000	1.000	<1.000	-
P. mirabilis 34/57	64.000	**16.000**	128.000	32.000	32.000	<1.000
***P. vulgaris* 8344**						
[a]Control	256.000	256.000	[b]**256.000**	256.000	128.000	[c]-
P. vulgaris 8344	<1.000	<1.000	**<1.000**	<1.000	<1.000	-
P. mirabilis 26/57	<1.000	<1.000	**<1.000**	<1.000	<1.000	-
P. mirabilis RMS 203	<1.000	<1.000	**<1.000**	<1.000	<1.000	-
P. rustigianii	<1.000	<1.000	**1.000**	<1.000	<1.000	-
P. myxofaciens	8.000	8.000	**8.000**	<1.000	<1.000	-

[a]Unadsorbed antiserum was used as control; [b]Data for homologous LPS are in bold; [c]not tested

a. *Proteus mirabilis* 26/57, *Proteus vulgaris* 8344 (O13) (Knirel *et. al*., 2011)



b. *Proteus myxofaciens* (O60) (Sidorczyk *et. al*., 2003)



c. *Providencia rustigianii* (O14) (Kocharova *et. al*., 2003)

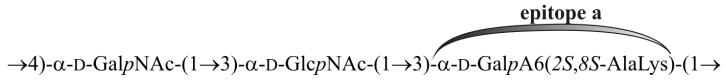


Figure 2. Structures of the O-polysaccharide of *P. mirabilis* 26/57 and *P. vulgaris* 8344 (O13) (a); *P. myxofaciens* (O60) (b) and *P. rustigianii* O14 (c).
The common epitopes are indicated by arcs. GalNAc, 2-acetamido-2-deoxy-D-galactose; Gal, galactose; GalA6(*2S*,*8S*-AlaLys), N^{ε}-[(*S*)-1-Carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine; GlcA6(*2S*,*8R*-AlaLys), amide of glucuronic acid with N^{ε}-[(*R*)-1-Carboxyethyl]-L-lysine; GlcNAc, 2-acetamido-2-deoxy-D-glucose.

O14 bacterial mass with O13 LPSs, also revealed a high level of similarities of *P. rustigianii* O14 LPS and three O13 LPSs (Table 1). Also this time, the similar banding patterns restricted to low-molecular mass LPS molecules of *P. rustigianii* O14, *P. mirabilis* RMS 203 and 26/57 LPSs were observed in a Western blot only for one antiserum, specific to *P. vulgaris* 8344 (Fig. 1b).

The last of the tested antigens, *P. myxofaciens*, differed the most in its serological specificity. After using this strain for the adsorption procedure, the highest levels of antibodies remained in each serum reacting with *P. mirabilis* 26/57, RMS 203 and *P. vulgaris* 8344 LPSs (Table 1). The biggest differences in the *P. myxofaciens* LPS specificity, compared to that of the other tested antigens, could be noticed with the *P. vulgaris* 8344 antiserum (Table 1, Fig. 1b). The *P. myxofaciens* LPS was the only antigen that in a Western blot did not show any reactions with core-specific antibodies (Fig. 1a, b, c), which suggests that its core region differs significantly from the core regions of the other tested LPSs. However, such an observation may have also resulted from a lower level of the core-specific antibodies in the *Proteus* O13 antisera when compared to *e.g.* the *P. myxofaciens* antiserum, for which other authors noted a reaction with fast migrating bands of *P. mirabilis* 26/57 (O13) LPS in a Western blot (Sidorczyk *et al*., 2003).

The *P. myxofaciens* and *P. rustigianii* O14 LPSs were selected for the study since they share a common fragment of OPSs: *2S*,*8S*/*R*-AlaLys with the OPSs of the *Proteus* spp. O13 LPSs. What is more, in *P. rustigianii* O14 and *Proteus* spp. O13 OPSs this fragment is attached to the α-D-GalA residue forming an amide (Shashkov *et al*., 1997; Kocharova *et al*., 2003). The contribution of the amide of α-D-GalA with lysine to serological cross-reactions was often described for different *Proteus* spp. LPSs. The amide was found to be a potential epitope in the OPSs of *P. mirabilis* 51/57 (O28), S1959 (O3a,b), G1 (O3a) LPSs or in T-antigen of the *P. mirabilis* R14/S1959 LPS as well as in the core regions of *P. penneri* 42 (O71), *P. mirabilis* 51/57 (O28) and R14 LPSs (Radziejewska-Lebrecht *et al*., 1995; Bartodziejska *et al*., 1996; Sidorczyk *et al*., 2002; Palusiak *et al*., 2014). These data encouraged us to check if the *2S*,*8S*/*R*-AlaLys or α-D-GalA6(*2S*,*8S*/*R*-AlaLys) fragments play a role of potential epitopes in *Proteus* spp. O13 LPSs.

The α-D-GalA6(*2S*,*8S*/*R*-AlaLys) (epitope a in Fig. 2) is responsible for the strong cross-reactivity of each tested *Proteus* O13 antiserum with *P. rustigianii* O14 LPS, since the remaining part of its OPS has nothing in common with the O13 O-antigen. Similarly, *2S*,*8R*-AlaLys (epitope b in Fig. 2) is the only fragment of the *P. myxofaciens* OPS common to the O13 OPS, and thus, it plays a role of a potential epitope contributing to the cross-reactivity of the *Proteus* O13 antisera with the *P. myxofaciens* LPS (Table 1, Fig. 1).

Both mentioned residues have been also shown to be responsible for the cross-reactivity of the anti-*P. rustigianii* O14 serum with the *P. mirabilis* O13 or *P. myxofaciens* LPSs, which confirms the serological data presented here (Kocharova et al., 2003). It is worth noting that in the previous published work, the importance of the AlaLys group in the serological specificity of the *P. mirabilis* O13 and *P. rustigianii* O14 OPSs was revealed (removing this group from both OPSs abolished or reduced their inhibiting activities, respectively). What is more, it was suggested that the absolute configuration of the 1-Carboxyethyl group did not play a crucial role in the recognition of antibodies (Kocharova *et al*., 2003). This observation was also confirmed by the current studies. All *Proteus* spp. O13 antisera exhibited strong cross-reactivity with the *P. rustigianii* O14 LPS (Table 1, Fig. 1) despite the fact that its OPS has the stereoisomer of AlaLys (*2S*,*8S*) different from that found in the O13 OPS (*2S*,*8R*). The dominance of α-D-GalA6(*2S*,*8R*-AlaLys) (epitope a in Figure 1) in the serological specificity of *Proteus* spp. O13 OPSs was also revealed by the lack of reactivity of the *P. mirabilis* 26/57 antiserum with the *P. mirabilis* 34/57 (O18) LPS. It is worth noting that the OPS of *P. mirabilis* 34/57 (O18) contains a fragment (β-D-Gal*p*-(1→3)-

β-D-Glc*p*NAc) similar to that of O13 OPS but without the α-D-GalA6(2*S*,*8R*-AlaLys) group (Knirel *et al.*, 2011).

High similarities between the titres in each serum (after or without its adsorption) observed in ELISA (Table 1) and between the patterns of slow migrating bands of O13 LPSs shown in the Western blot (Fig. 1), revealed that *P. mirabilis* RMS 203 presents the same O serotype as *P. mirabilis* 26/57 and *P. vulgaris* 8344. Thus, the O13 serogroup was completed with another representative – *P. mirabilis* RMS 203. The data presented here indicate that the O13 LPS is a serologically important antigen with a fragment common not only to many representatives of the *Proteus* genus but also to the *P. rustigianii* O14 strains.

Finding that the LPSs of different species from the *Proteeae* tribe cross-react with the *Proteus* O13 antisera is expected to be of high importance in the future search for vaccine antigens common to different bacteria species.

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