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The amide of galacturonic acid with lysine as an immunodominant component of the lipopolysaccharide core region from *Proteus penneri* 42 strain*

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Most *Proteus* lipopolysaccharides (LPSs) contain uronic acids or their amides with different amino acids, which together with other negatively charged components account for the acidic character of such LPS molecules. Previous studies have shown the significance of an amide of galacturonic acid with lysine [D-GalA(L-Lys)] for serological specificity of O-antigens from few *P. mirabilis* strains. In this work, the immunodominant role of GalALys was indicated for the *P. penneri* 42 LPS core region. The studies also showed the serological identity of core oligosaccharides from *P. penneri* 42 (O71), *P. mirabilis* 51/57 (O28) and R14/S1959 strains.

Key words: amide of galacturonic acid with lysine, anti-conjugate serum, core region, lipopolysaccharide, *Proteus*

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INTRODUCTION

Proteus bacteria, including *P. mirabilis* and *P. penneri* species, are human opportunistic pathogens causing different types of infections in preferable conditions, but the most frequent are urinary tract infections (UTIs) (Różalski *et al*., 2012). UTIs are often persistent and can lead to complications like pyelonephritis, as well as bladder or kidney stone formation. The latter process is mainly a result of the activity of urease, which catalyses the hydrolysis of urea to ammonia and carbon dioxide. The presence of ammonia leads to the elevation of pH and alkalization of urine, which in turn induces the precipitation of magnesium and calcium ions, and the formation of urinary stones, containing struvite and carbonate apatite. This phenomenon can be modified by the O-specific polysaccharide part of lipopolysaccharides (LPSs). Depending on the chemical structure of these polysaccharides and their ability to bind cations Ca^{2+} and Mg^{2+} the crystallization of struvite and carbonate apatite may be enhanced or inhibited. The negatively charged groups found in bacterial polysaccharides including LPSs play an important role in this process (Torzewska *et al*., 2003). It was found that negatively charged components like hexuronic acids, non-sugar carboxylic acids or phosphate groups are responsible for the acidic character of approximately 90% of *Proteus* sp. O-polysaccharides (OPSs) (Knirel *et al*., 2011). Hexuronic acids, including galacturonic acid (GalA) are often found in *Proteus* sp. OPSs, where they can be amidated with amino acids such as: L-alanine, L-serine and L-lysine (Lys; the most common) (Knirel *et al*., 2011). The GalA residue is present also in the *Proteus* LPS core region: in the proximal part of oligosaccharide — α-linked GalA (in all structurally tested core oligosaccharides) and β-linked (mainly in *P. mirabilis* strains), as well as in the distal part — as an amide of GalA and Lys. The latter was demonstrated in one *P. penneri* strain and two *P. mirabilis* strains (Vinogradov *et al*., 2002; Vinogradov 2011). In the previously conducted studies GalALys appeared to play a crucial role in serological cross-reactions of *P. mirabilis* O28, S1959 LPSs and LPS from *P. mirabilis* R14/S1959 (a transient form of *P. mirabilis* S1959 containing T-antigen as a polysaccharide chain) with hetorologous polysaccharide-specific antisera (Radziejewska-Lebrecht *et al*. 1995; Bartodziejska *et al*., 1996). However, the above mentioned studies were focused on epitope characterization of the O-specific or T-specific (R14/S1959) polysaccharides and used polyclonal rabbit antisera against the *P. mirabilis* O28, S1959 and *P. mirabilis* R14/S1959 strains. These sera included a high level of polysaccharide-specific antibodies, which was revealed *e.g.* by the weak reaction of *P. mirabilis* R14/S1959 antiserum with the core oligosaccharide fraction from homologous LPS and by the strong reaction with its T-polysaccharide fraction (Bartodziejska *et al*., 1996). The presence of the GalALys amide in the LPS core region of *P. penneri* 42 strain encouraged us to obtain the serum against the core oligosaccharide (OS) of the LPS of these bacteria (anti-conjugate serum) and check if the GalALys fragment is also important for the immunospecificity of the LPS core region.

MATERIALS AND METHODS

The set of bacterial strains. *P. penneri* 42 (O71) is a clinical isolate from the patients from Toronto (Canada)

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Abbreviations: Ara*p*4N, 4-amino-4-deoxy-L-arabinopyranose; ELISA, enzyme-linked immunosorbent assay; GalA, galacturonic acid; GalALys, amide of GalA with the α-aminogroup of L-lysine; GalNAc, 2-acetamido-2-deoxy-D-galactose; Glc, glucose; GlcNGly, 2-deoxy-2-glycylamido-D-glucose; Hep, L-*glycero*-D-*manno*-heptose; DD-Hep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; LPS, lipopolysaccharide; OPS, O-poly-saccharide; OS, oligosaccharide; *P*Etn, 2-aminoethyl phosphate; UTIs, urinary tract infections.

but its isolation source remains unknown. This strain together with the other 26 ones: *P. penneri* 1 (O72a), *P. penneri* 2 (O66), 4 (O72a,b), 7 (O61), 8 (O67), 11, 12 (O58), 13 (R form), 14 (O59), 15 (O52), 16 (O17), 17 (O8), 18 (O17), 19 (O64a,b,c), 22 (O63), 25 (O69), 26 (O31a), 27 (O64a,b,c), 28 (O31a,b), 31 (O19a,b), 34 (O65), 37 (R form), 39, 40 (O64a,b,d), 41 (O62), 44 (R form) were kindly provided by Professor D. J. Brenner, Center for Diseases Control and Prevention in Atlanta (USA). *P. penneri* 60 (O70), 63 (O68), 75 (O73a,b) and *P. penneri* 71 (O64a,c,e) strains were isolated from the urine of patients in hospitals in Łódź and Warsaw (Poland), respectively. The other strains were kindly provided: *P. penneri* 47 (O59) by Dr. E. Falsen, Department of Clinical Bacteriology, Göteborg (Sweden); *P. penneri* 77 (O65) and 93 (O67) by Dr. B. Senior, Department of Medical Microbiology in Dundee (UK); *P. penneri* 103 (O73a,c), 104 (O61), 107, 112 (O8) and *P. penneri* 124 (R form) by Dr. B. Holmes, Central Public Health Laboratory in London (UK); *P. penneri* 133 (O61) by Dr. G. Giammanco, Institute of Hygiene and Prevention Medicine in Catania (Italy). *P. mirabilis* strain G1 (O3a) was kindly provided by J. Gmeiner (Institute for Microbiology and Genetics, Darmstadt, Germany), *P. mirabilis* 51/57 (O28) comes from the Czech National Collection of Type Cultures, Institute of Epidemiology and Microbiology, Praque, Czech Republic. The above mentioned strains along with *P. mirabilis* S1959 (O3a,b) and its mutants: R110 and R14 (a transient-like form) belong to the collection of the Department of General Microbiology, University of Łódź (Poland), where they are stored in a glycerol mixture at –80°C. The bacteria were grown for 18 h under aerobic conditions in liquid nutrient broth containing 1% glucose (BTL, Poland) at 37°C, killed with 1% phenol, centrifuged, washed twice with water and lyophilized to dried bacterial cells.

Lipopolysaccharide. The LPSs were extracted from dried bacterial cells by the phenol-water procedure according to the method by Westphal (Westphal & Jann, 1965) and purified with aqueous 50% trichloroacetic acid. Alkali-treated LPSs used for the sera adsorption were prepared as described in detail elsewhere (Palusiak *et al*., 2008).

After the degradation of *P. penneri* 42 LPS with 1.5% acetic acid (100°C for 1 h) lipid A was separated from a water-soluble carbohydrate portion by centrifugation (13 000 × *g*, 20 min., 4°C). The sugar portion was then separated on a Bio-Gel P-10 column (1.6 × 100 cm, equilibrated with 0.05 M pyridine/acetic acid buffer, pH 5.6). Eluates were monitored with a Knauer differential refractometer. The fractions were eluted, freeze-dried and checked by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) run on a Kratos Kompact-SEQ instrument.

***P. penneri* 42 core oligosaccharide conjugate.** The conjugation of the *P. penneri* 42 core oligosaccharide with diphtheria toxoid was performed by the method of H. J. Jennings and C. Ługowski based on the reaction of reductive amination, which was described in detail elsewhere (Jennings & Ługowski, 1981).

The *P. penneri* 42 anti-conjugate serum was gained by the immunization of New Zealand white rabbits according to the procedure described in detail elsewhere (Palusiak *et al*., 2008).

Serological assays. Purified LPS samples were tested with rabbit antisera in an enzyme-linked immunosorbent assay (ELISA), Western blot procedure after sodium dodecyl sulfate polyacrylamide gel electrophoresis with non-adsorbed antisera and/or antisera adsorbed with selected alkali-treated LPSs. All assays were performed as previously described (Sidorczyk *et al*., 2002; Palusiak *et al*., 2008).

RESULTS AND DISCUSSION

In this work, the serological specificity of the LPS core region from *P. penneri* 42 LPS was characterized. The first step of the study included the preparation of the *P. penneri* 42 diphtheria toxoid-core oligosaccharide conjugate, which was used as an immunogen in the process of obtaining the anti-conjugate serum. The serum, containing the antibodies specific to OS of homologous LPS, was checked in ELISA with a set of 45 *Proteus* LPS preparations. These antigens were selected for the study on the grounds of previously conducted investigations and come from strains representing many *Proteus* O-serogroups. The tested antiserum reacted only with five LPSs [*P. penneri* 42 (O71), *P. mirabilis* 51/57 (O28), G1 (O3a), S1959 (O3a,b) and *P. mirabilis* R14/S1959]. As shown in Table 1, only one LPS, *P. mirabilis* R14/S1959, reacted to the same titer as *P. penneri* 42 LPS, *P. mirabilis* 51/57 (O28) LPS reacted with heterologous antibodies similarly to the homologous antigen, meanwhile the remaining two LPSs bound the immunoglobulins much weaker. In the Western blot technique, after the separation of LPS samples in sodium dodecyl sulfate polyacrylamide gel electrophoresis, *P. penneri* 42 anti-conjugate serum recognized fast migrating bands (restricted to core-lipid A molecules) of *P. penneri* 42, *P. mirabilis* 51/57 and R14/S1959 LPSs, which core regions seem to be identical. Moreover, the tested serum bound to slow migrating bands (corresponding to high-molecular-mass LPS species containing a core-lipid A moiety substituted with O-polysaccharide chains) of *P. mirabilis*

Table 1. ELISA data of the reactivity of *Proteus* sp. LPSs with adsorbed anti-conjugate serum against *P. penneri* 42 strain[a,b]
[a]Non-adsorbed antiserum was used as control; [b]Data for homologous LPS are bolded

P. penneri 42 anti-conjugate serum	Reciprocal titer of adsorbed antiserum for LPS from strains				
	P. penneri	*P. mirabilis*			
	42	51/57	G1	S1959	R14/S1959
Control	**8.000**	4.000	2.000	2.000	8.000
P. penneri 42	**<500**	**<500**	**<500**	**<500**	**<500**
P. mirabilis 51/57	<500	<500	<500	<500	<500
P. mirabilis G1	4.000	2.000	<500	<500	4.000
P. mirabilis S1959	4.000	2.000	<500	<500	4.000
P. mirabilis R14/S1959	<500	<500	<500	<500	<500

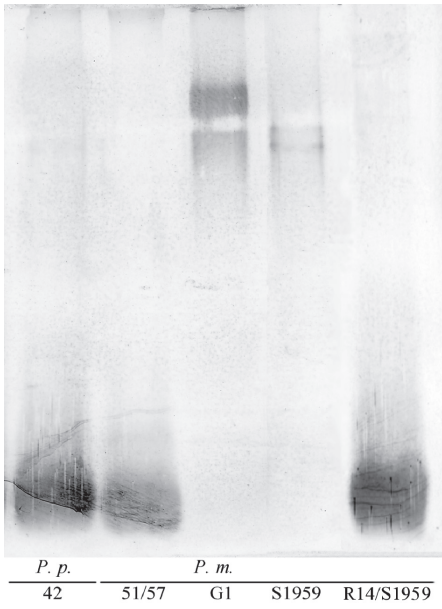


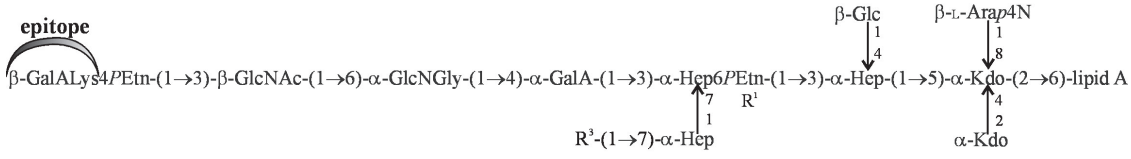
Figure 1. Western blot of *Proteus* sp. LPSs with serum against the core oligosaccharide of the *P. penneri* 42-diptheria toxoid conjugate.

G1 and S1959 LPS preparations. Similarly to the results obtained in ELISA (Table 1), the last two reactions were weaker than those observed for the other tested LPSs. To confirm the similarities in serological specificity of the used antigens the adsorption procedure of the antiserum by the tested LPS preparations was performed. The reactivity of the adsorbed antiserum (compared to the non-adsorbed one) was checked in ELISA with all used LPSs. Adsorption of antiserum by alkali-treated LPSs of *P. penneri* 42, *P. mirabilis* 51/57 and R14/S1959 strains completely abolished the serum reactions with all used antigens (Table 1), which confirmed the identical binding patterns observed for these three LPSs in the Western blot technique (Fig. 1). After the serum adsorption by *P. mirabilis* G1 and S1959 LPSs, there was no reactivity in these systems, whereas the reactivity titers for *P. penneri* 42, *P. mirabilis* 51/57 and *P. mirabilis* R14/S1959 LPSs were slightly lower compared to the control systems.

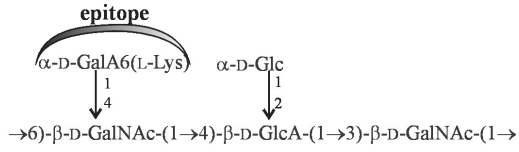
To summarize, the core oligosaccharides of *P. penneri* 42 and *P. mirabilis* 51/57 LPSs have a common major epitope responsible for the strong reactions of these LPSs with homologous and heterologous antibodies, respectively. Such outcomes stay in agreement with the previously published results of chemical analysis, which showed the structural identity of the LPS core regions from *P. penneri* 42 and *P. mirabilis* 51/57 strains (Vinogradov *et al.*, 2002). *P. mirabilis* G1 and S1959 OPSs contain a minor common epitope, which is also localized in the LPS core regions of *P. penneri* 42 and *P. mirabilis* 51/57 LPSs.

Interestingly, these studies revealed that there is another *Proteus* LPS (*P. mirabilis* R14/S1959), which probably possesses a major epitope common with the OS of *P. penneri* 42 and probably *P. mirabilis* 51/57 LPSs. It was found by the reactivity of the *P. penneri* 42 anti-conjugate serum with R14/S1959 LPS, which was identical in the reactions observed in ELISA and in Western blot for *P. penneri* 42 LPS. What is most important, this LPS used for the adsorption of the *P. penneri* 42 anti-conjugate serum abolished the reactions with all tested LPSs (*P. penneri* 42, *P. mirabilis* 51/57, G1 and S1959), which is the best proof of the serological identity of the OS from *P. penneri* 42 and *P. mirabilis* R14/S1959 LPSs (Table 1). The previous serological studies using antiserum against *P. mirabilis* O28 strain suggested only the presence of a common epitope (probably GalALys) in the LPS of *P. mirabilis* O28 and the core region of *P. mirabilis* R14/S1959 LPS, but they did not reveal the identical serospecificity of the core regions from these two LPSs (Radzie-

a. *P. penneri* 42 (O71) and *P. mirabilis* 51/57 (O28) R^3=H (Vinogradov *et al.*, 2002)



b. *P. mirabilis* S1959 (O3a,b) (Knirel *et al.*, 2011)



c. *P. mirabilis* G1(O3a) (Sidorczyk *et al.*, 2002)

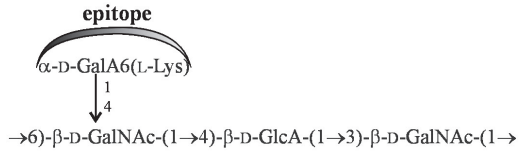


Figure 2. Structures of: the core region of *P. penneri* 42 and *P. mirabilis* 51/57 LPS (a); O-polysaccharide of *P. mirabilis* S1959 (b) and *P. mirabilis* G1 (c).
A common epitope is indicated by arcs. Ara*p*4N, 4-amino-4-deoxy-L-arabinopyranose; GalA, galacturonic acid; GalALys, amide of D-GalA with the α-aminogroup of L-lysine; GalNAc, 2-acetamido-2-deoxy-D-galactose; Glc, glucose; GlcNGly, 2-deoxy-2-glycylamido-D-glucose; Hep, L-*glycero*-D-*manno*-heptose; DD-Hep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-v-*manno*-oct-2-ulosonic acid; *P*Etn, 2-aminoethyl phosphate.

jewska-Lebrecht *et al*., 1995; Bartodziejska *et al*., 1996). It is worth mentioning that although *P. mirabilis* R14/S1959 strain is a transient mutant of S1959 strain, its polysaccharide T-antigen (similar to the T1 chain of *Salmonella* Friedenau LPS) is characterized by the structure completely different from the structure of the O-antigen of its parental strain. At the same time, the T-antigen of *P. mirabilis* R14/S1959 is identical in its structure with the OPS of *P. penneri* 42 LPS (Bartodziejska *et al*., 1996; Knirel *et al*., 2011). Combining this fact with the results of the serological studies, showing the identity of serological specificity of the core regions from *P. penneri* 42 and *P. mirabilis* R14/S1959 LPSs, it seems to be probable that both LPSs have much in common not only in their polysaccharide parts but also in the core regions. It is interesting since such huge structural and/or serological similarities between LPSs of the strains from two different species are rather uncommon.

Comparing the structures of OS from *P. penneri* 42 and *P. mirabilis* 51/57 LPSs and of OPS from *P. mirabilis* G1 and S1959 LPSs (the structures differ only in the lateral D-Glc residue present in *P. mirabilis* S1959 LPS), it has been revealed that they all share an amide of galacturonic acid with lysine as a distal component of the core region of the first mentioned LPSs (*P. penneri* 42 and *P. mirabilis* 51/57) and as a lateral branch in OPS of the others (Fig. 2) (Knirel *et al*., 2011; Sidorczyk *et al*., 2002; Radziejewska-Lebrecht *et al*., 1995; Vinogradov *et al*., 2002). These fragments differ only in their conformation (β-linked in *P. penneri* 42 and *P. mirabilis* 51/57 core regions and α-linked in *P. mirabilis* G1 and S1959 OPSs). As showed by Radziejewska-Lebrecht et *al*. (1995), using the synthetic glycopolymers containing amides of D-GalA with different amino acids, including lysine, the immunodominant part of the mentioned epitope is probably an $NH_2(CH_2)_4$ group of lysine rather than the whole residue. According to these data, the α or β type of linkage may not play an important role in the immunospecificity of the amide.

There are two reasons supporting the hypothesis of the GalALys domination in the specificity of the *P. penneri* 42 LPS core region. Firstly, *P. penneri* 42 anti conjugate serum reacted in ELISA only with LPSs possessing the mentioned amide in their structures. Secondly, the other studies indicated that the majority of antibodies in the core-specific sera recognize the most distal fragments of the LPS core regions (Palusiak *et al*., 2008; Palusiak & Sidorczyk, 2010), and the GalALys amide represents this type of residues.

Indicating such immunogenic epitopes as GalALys, common not only for the LPS core regions but also for its O-polysaccharides from different species, should be found important because of the potential application of such fragments as synthetic antigens used for obtaining an appropriate protective serum.

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