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Classification of *Proteus mirabilis* TG 115 and CCUG 10701 into the *Proteus* O23 serogroup based on chemical and serological studies of O-polysaccharides

Agnieszka Zabłotni[1], Andrei V. Perepelov[2], Katarzyna Kołodziejska[1], Krystyna Zych[1], Yuriy A. Knirel[2] and Zygmunt Sidorczyk[1]

[1] Department of General Microbiology, Institute of Microbiology and Immunology, University of Łódź, Łódź, Poland
[2] N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russian Federation

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Abstract

Introduction: Bacteria of the genus *Proteus* are a common cause of urinary tract infections. The O-polysaccharide (OPS) chain of their lipopolysaccharide (LPS) defines the serological specificity of strains. Based on the OPS structures and the immunospecificity of the LPS, *Proteus* strains have been classified into 74 O-serogroups.
Materials and Methods: The OPS of *P. mirabilis* TG 115 was obtained by mild acid degradation of the LPS and studied by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. Antisera were raised by immunization of rabbits with heat-killed bacteria. Serological studies were performed using enzyme immunosorbent assay, passive immunoheamolysis, inhibition experiments, absorption of O-antisera, and Western blot.
Results: The following structure of the *P. mirabilis* TG 115 OPS was established:
→2)-β-D-Gal*p*A-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→
The same structure has been reported previously for the O-polysaccharides of *P. mirabilis* CCUG 10701 (O74) and *P. mirabilis* 41/57 (O23), except that they contain O-acetyl groups in non-stoichiometric quantities. Serological studies showed the antigenic identity of the three strains and their close serological relatedness to *P. vulgaris* 44/57.
Conclusions: Based on the OPS structures and serological data, it is suggested to classify *P. mirabilis* 41/57, TG 115, and CCUG 10701 into one subgroup and *P. mirabilis* 42/57 and *P. vulgaris* 43/57 and 44/57 into another subgroup of the *Proteus* O23 serogroup.

Key words: *Proteus*, lipopolysaccharide, O-polysaccharide, O-serogroup, serological classification.

Abbreviations: EIA – enzyme immunosorbent assay, LPS – lipopolysaccharide, OPS – O-polysaccharide, PIH – passive immunohemolysis, NMR – nuclear magnetic resonance, CCUG – Culture Collection of the University of Goeteborg.

Corresponding author: Prof. Zygmunt Sidorczyk, Ph.D., Department of General Microbiology, Institute of Microbiology and Immunology, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland, tel.: +48 42 635-44-67, fax: +48 42 665-58-18, e-mail: zsidor@biol.uni.lodz.pl

INTRODUCTION

The Gram-negative bacilli of the genus *Proteus* are currently divided into five named species, i.e. *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens*, and *P. hauseri*, and the three unnamed *Protues* genomospecies 4, 5, and 6 [6, 7]. These bacteria are facultative pathogens causing urinary tract infections, often with severe complications such as catheter obstruction and the formation of bladder and kidney stones [13, 21]. A number of virulence factors of *Proteus* that mediate the infection processes have been described, including fimbriae, swarming phenomenon, urease, proteases, and lipopolysaccharide (LPS, endotoxin) [11]. The serological specificity of *Proteus* bacilli, similarly to other genera of the *Enterobacteriaceae* family, is defined by the chemical structure of the O-polysaccharide (OPS, O-antigen) chain of the outer membrane LPS. The first commonly accepted scheme of serological classification of *Proteus* bacilli was made by Kauffmann [2] and contained two

species only: *P. mirabilis* and *P. vulgaris*. Later, new O-serogroups containing strains of other *Proteus* species were created, i.e. *P. penneri* [4, 16, 18, 25], *P. myxofaciens* [15], and the unnamed *Proteus* genomospecies 4, 5, and 6 [26]. The above scheme is not complete, even for the species *P. mirabilis* and *P. vulgaris*, because Larsson et al. [5] and Penner and Hennesy [8] supplemented it by 6 and 11 new *Proteus* O-serogroups, respectively. In this work we report the chemical and serological studies of LPSs isolated from a hitherto unclassified strain, *P. mirabilis* TG 115, and propose a new classification of this and related *Proteus* strains into serogroup O23 based on the data obtained.

MATERIALS AND METHODS

Bacterial strains

P. mirabilis strain TG 115 was kindly provided by Prof. Penner (Department of Medical Genetics, University of Toronto, Canada). *P. mirabilis* OB, strain CCUG 10701, was obtained from the Culture Collection of the University of Goeteborg (CCUG), Goeteborg, Sweden. Thirty-nine strains of *P. mirabilis* and 27 strains of *P. vulgaris* were from the Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic. Twenty-four strains of *P. penneri*, three strains of *Proteus* genomospecies, and one strain of *P. hauseri* were kindly provided by Dr. O'Hara and Dr. Brenner (Centers for Disease Control and Prevention, Atlanta, Georgia, USA).

Cultivation of bacteria, isolation and saponification of theLPSs

The bacteria were cultivated under aerobic conditions on nutrient broth (BTL, Łódź, Poland). The bacterial mass was harvested at the end of the exponential growth phase, centrifuged, washed with water, and lyophilized.

The LPSs were isolated from the dried bacterial cells by phenol/water extraction [22] and purified by treatment with cold aqueous 50% CCl_3CO_2H followed by dialysis of the supernatant [28]. The LPSs were treated with 0.25 M NaOMe in absolute MeOH at 37°C for 15 h [23] and the product was dissolved in an excess of water and lyophilized.

Preparation of the OPS

Delipidation of the *P. mirabilis* TG 115 LPS was performed by mild acid degradation with aqueous 2% HOAc at 100°C until precipitation of a lipid. The precipitate was removed by centrifugation (13,000×g, 20 min) and the supernatant was fractionated by gel-permeation chromatography on a column (56×2.6 cm) of Sephadex G-50 (Amersham Biosciences, Sweden) in 0.05 M pyridinum acetate buffer (pH 4.5), monitored by a differential refractometer (Knauer, Germany). The yield of the high-molecular-mass OPS of *P. mirabilis* TG 115 was 21% of the LPS weight.

Nuclear magnetic resonance spectroscopy

Prior to measurements, the samples were freeze--dried twice from D_2O. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker DRX-500 spectrometer (Germany) for solutions in D_2O at 30°C using internal acetone (δ_H 2.225, δ_C 31.45) as the reference. Standard Bruker software (XWINNMR 2.6) was used to acquire and process the NMR data. ^{1}H- and ^{13}C-NMR chemical shifts of the *P. mirabilis* TG 115 OPS are tabulated in Table 1.

Rabbit antisera and serological assays

Polyclonal O-antisera were obtained by immunizing rabbits (two for one antiserum) with heat-inactivated bacteria of *P. mirabilis* TG 115, CCUG 10701, and *P. vulgaris* PrK 44/57 (O23) according to a published procedure [27].

RESULTS AND DISCUSSION

Elucidation of the OPS structure

The OPS was released by mild acid degradation of LPS isolated from *P. mirabilis* TG 115 cells by the phe-

Table 1. ^{1}H- and ^{13}C-NMR data of the OPS of *P. mirabilis* TG 115 (δ, ppm)

Sugar residue	Nucleus	Atom number					
		1	2	3	4	5	6 (6a,6b)
→2)-β-D-Gal*p*A-(1→	^{1}H	4.57	3.70	3.72	4.17	4.27	
	^{13}C	103.50	78.30	75.00	71.30	75.20	173.40
→3)-α-D-Gal*p*NAc-(1→	^{1}H	4.95	4.30	4.00	4.37	4.36	3.73
	^{13}C	100.00	49.60	78.50	69.30	71.70	61.60
→4)-α-D-Gal*p*A-(1→	^{1}H	5.38	3.90	3.98	4.41	4.42	
	^{13}C	101.80	69.50	69.70	79.50	71.30	173.60
→3)-β-D-Glc*p*NAc-(1→	^{1}H	4.83	3.76	3.73	3.65	3.38	3.71, 3.89
	^{13}C	102.90	55.70	83.70	71.90	76.70	61.90

The chemical shifts for the N-acetyl groups are δ_H 1.98 and 2.10; δ_C 23.3 and 24.1 (Me), 175.2 and 175.8 (CO).

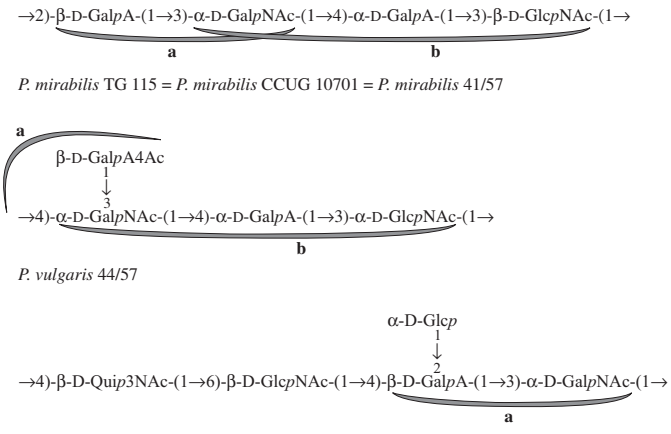

Fig. 1. Structures of the O-polysaccharides of *P. mirabilis* TG 115 (this study), *P. mirabilis* CCUG 10701 (OB) [10], *P. mirabilis* O23 (41/57) [1, 12], *P. vulgaris* O23 (44/57) [9], and *Proteus* genomospecies 4 [26]. O-Acetyl groups in the OPS of *P. mirabilis* CUG 10701 (OB) are not shown.

nol/water procedure. Structural studies, including chemical and NMR spectroscopic techniques as described in detail earlier [10], showed that the OPS has a linear tetrasaccharide repeating unit and enabled determination of its structure as shown in Fig. 1.

Comparison of the NMR spectroscopy data (Table 1) with published data [1, 10, 12, 19] indicated that the *P. mirabilis* TG 115 OPS structure is identical to that of the O-deacetylated polysaccharides of *P. mirabilis* CCUG 10701 of serogroup O74 [10] as well as *P. mirabilis* PrK 41/57 [1, 12] and 7570 [19] of serogroup O23 reported earlier. The initial O-polysaccharides of these bacteria differ by the presence of non-stoichiometric O-acetyl groups, which occupy position 6 of GlcNAc (~50%) and position 4 of β-GalA (~60%) in strain CCUG 10701 or are located at an undetermined position(s) in a lesser quantity in strains PrK 41/57 and 7570.

The related polysaccharides of *P. mirabilis* PrK 42/57 and *P. vulgaris* PrK 43/57 and PrK 44/57 have the same sugar composition and contain an O-acetyl group at position 4 of β-GalA (50–80%), but their interior tetrasaccharide repeating units are branched [9, 12] (Fig. 1).

Serological studies

LPS from 94 strains representing all the *Proteus* O-serogroups were tested with polyclonal rabbit O-antiserum against *P. mirabilis* TG 115 in passive immunoheamolysis (PIH) and enzyme immunosorbent assay (EIA). Only seven LPSs cross-reacted, namely *P. mirabilis* CCUG 10701 (OB), G1 (O3), 41/57 (O23), and 49/57 (O26), *P. vulgaris* 44/57 (O23), *P. penneri* 42 (O71), and *Proteus* genomospecies 4 (O56).

P. mirabilis TG 115 O-antiserum as well as *P. mirabilis* CCUG 10701 O-antiserum cross-reacted strongly with *P. mirabilis* TG 115, CCUG 10701, and 41/57 LPS (Table 2).

Table 2. Reactivity of *Proteus* O-antisera against *Proteus* LPS

LPS from strain	Reciprocal titer for the LPS in		Minimal inhibitory dose (ng) of the LPS in	
	PIH	EIA	PIH	EIA
P. mirabilis TG 115 O-antiserum				
P. mirabilis TG 115	*51200*	*1024000*	*2*	*2*
P. mirabilis CCUG 10701	51200	1024000	2	4
P. mirabilis O23 (41/57)	51200	1024000	2	4
P. vulgaris O23 (44/57)	6400	64000	20	80
P. mirabilis O3	3200	16000	>1000	>1000
P. mirabilis O26	3200	32000	>1000	>1000
P. penneri O71	3200	16000	>1000	>1000
Proteus genomospecies 4 (O56)	1600	4000	>1000	>1000
P. mirabilis CCUG 10701 O-antiserum				
P. mirabilis TG 115	25600	256000	1	2
P. mirabilis CCUG 10701	*25600*	*256000*	*1*	*2*
P. mirabilis O23 (41/57)	25600	128000	1	4
P. vulgaris O23 (44/57)	6400	32000	20	40
P. mirabilis O3	3200	16000	>1000	>1000
P. mirabilis O26	3200	16000	>1000	>1000
P. penneri O71	3200	16000	>1000	>1000
Proteus genomospecies 4 (O56)	1600	4000	>1000	>1000
P. vulgaris PrK 44/57 (O23) O-antiserum				
P. mirabilis TG 115	3200			
P. mirabilis CCUG 10701	3200			
P. mirabilis O23 (41/57)	3200			
P. vulgaris O23 (44/57)	*25600*			

LPS and alkali-treated LPS were used as antigens in EIA and PIH, respectively. Data for homologous LPS are italicized.

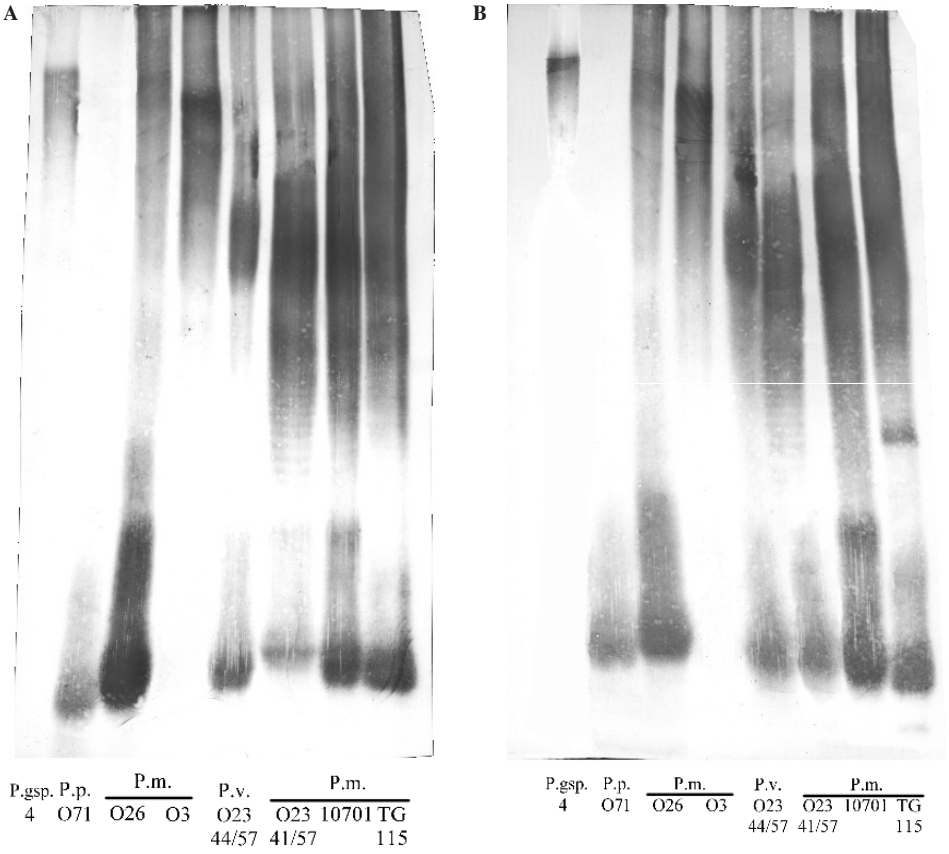

Fig. 2. Western blot of *Proteus* LPS with the O-antisera against *P. mirabilis* TG 115 (**A**) and *P. mirabilis* CCUG 10701 (**B**). *P.m., P.v., P.p., and P*. gsp. stand for *P. mirabilis*, *P. vulgaris*, *P. penneri*, and *Proteus* genomospecies, respectively.

A slightly weaker reaction was observed with the LPS of *P. vulgaris* 44/57, whereas all other LPSs reacted markedly weaker. The specificity of the reactions was confirmed by their inhibition in both PIH and EIA (Table 2). Again, the first three LPSs were the strongest inhibitors (minimal inhibiting dose: 1–4 ng) and the inhibitory activity of *P. vulgaris* 44/57 LPS was slightly weaker (20–40 ng; Table 2). The other cross-reactive LPSs were poor inhibitors (>1000 ng).

In Western blot, both *P. mirabilis* TG 115 and CCUG 10701 O-antisera clearly recognized high-molecular-mass OPS-containing LPS species as well as low--molecular-mass LPS species restricted to the core-lipid A moiety from *P. mirabilis* TG 115, CCUG 10701, 41/57, and O26 and *P. vulgaris* 44/57 (Fig. 2a, b). Antibodies bound also to high-molecular-mass LPS species from *P. mirabilis* O3 and *Proteus* genomospecies 4 and to low-molecular-mass LPS species from *P. penneri* O71.

Absorption of both O-antisera with each of the *P. mirabilis* TG 115, CCUG 10701, and 41/57 LPSs completely abolished their reactivity with all the tested antigens (Table 3). Absorption with *P. vulgaris* 44/57 decreased only the O-antisera titers with *P. mirabilis* TG 115, CCUG 10701, and 41/57 LPS, whereas the reactivity with the other antigens was abolished. *P. mirabilis* O3 and *P. penneri* O71 LPS reacted similarly, i.e. they removed from both O-antisera cross-reacting antibodies against these two LPSs, decreased the reactions with the other tested antigens, but did not influence the reactivity with *Proteus* genomospecies 4 LPS. The reactivity with *P. mirabilis* O3 and O26 and with *P. penneri* O71 LPS was completely abolished when O-antisera were absorbed with *P. mirabilis* O26 LPS. Absorption with *Proteus* genomospecies 4 LPS decreased the reactivity with the first four cross-reactive LPSs listed in Table 3 (*P. mirabilis* TG 115, CCUG 10701, and 41/57 and *P. vulgaris* 44/57) and had no influence on the reactivity of the three other heterologous antigens.

Cross-reactivity of O-antiserum against *P. vulgaris* 44/57 with the LPS of *P. mirabilis* 41/57 was demonstrated earlier [12] and confirmed in this study, as well as its similarly marked reactivity with *P. mirabilis* TG 115 and CCUG 10701 LPS (Table 2). Absorption of *P. vulgaris* 44/57 O-antiserum with each of the three cross-reactive LPSs abolished the reactivities with all of them and significantly decreased the reactivity with *P. vulgaris* 44/57 LPS (Table 3).

These data show the serological identity of *P. mirabilis* TG 115, CCUG 10701, and 41/57 LPS and their close serological relatedness to *P. vulgaris* 44/57 LPS.

Table 3. PIH of the alkali-treated *Proteus* LPS with absorbed *Proteus* O-antisera

O-antisera absorbed with the alkali-treated LPS from *Proteus* strain	Reciprocal titer of absorbed O-antisera for the alkali-treated LPS from *Proteus* strains							
	P. mirabilis			*P. vulgaris*	*P. mirabilis*		*P. penneri*	*Proteus* genomospecies
	TG 115	CCUG 10701	O23 (41/57)	O23 (44/57)	O3	O26	O71	4
P. mirabilis TG 115 O-antiserum								
Control	51200	51200	51200	6400	3200	3200	3200	1600
P. mirabilis TG 115	<100	<100	<100	<100	<100	<100	<100	<100
P. mirabilis CCUG 10701	<100	<100	<100	<100	<100	<100	<100	<100
P. mirabilis O23 (41/57)	<100	<100	<100	<100	<100	<100	<100	<100
P. vulgaris O23 (44/57)	12800	12800	12800	<100	<100	<100	<100	<100
P. mirabilis O3	25600	25600	25600	3200	<100	800	<100	1600
P. mirabilis O26	25600	25600	25600	1600	<100	<100	<100	1600
P. penneri O71	25600	25600	25600	3200	<100	800	<100	1600
Proteus genomospecies 4 (O56)	25600	25600	25600	3200	3200	3200	3200	<100
P. mirabilis CCUG 10701 O-antiserum								
Control	25600	25600	25600	6400	3200	3200	3200	1600
P. mirabilis TG 115	<100	<100	<100	<100	<100	<100	<100	<100
P. mirabilis CCUG 10701	<100	<100	<100	<100	<100	<100	<100	<100
P. mirabilis O23 (41/57)	<100	<100	<100	<100	<100	<100	<100	<100
P. vulgaris O23 (44/57)	6400	6400	6400	<100	<100	<100	<100	<100
P. mirabilis O3	12800	12800	12800	1600	<100	800	<100	1600
P. mirabilis O26	12800	12800	12800	1600	<100	<100	<100	1600
P. penneri O71	12800	12800	12800	1600	<100	800	<100	1600
Protues genomospecies 4 (O56)	12800	12800	12800	1600	3200	3200	3200	<100
P. mirabilis O23 (44/57) O-antiserum								
Control	3200	3200	3200	25600				
P. mirabilis TG 115	<100	<100	<100	6400				
P. mirabilis CCUG 10701	<100	<100	<100	6400				
P. mirabilis O23 (41/57)	<100	<100	<100	6400				
P. vulgaris O23 (44/57)	<100	<100	<100	<100				

Epitope characterization and classification of strains to serogroup O23

The serological data are in agreement with the OPS structures of the *Proteus* LPSs studied (Fig. 1). Thus the serologically identical LPSs of *P. mirabilis* TG 115 (this study), CCUG 10701 [10], and 41/57 [1] have the same OPS carbohydrate backbone. The O-polysaccharides differ in their O-acetylation patterns, but the O-acetyl groups were found to be unimportant in the present serological study.

The cross-reactivity of the three *P. mirabilis* LPSs and that of *P. vulgaris* 44/57, which have different interior OPS repeating units, could be accounted for by the identity of the terminal non-reducing repeating units, which significantly contribute to the immunospecificity [9]. Comparison of their structures suggests that the corresponding common O-factor "a" is associated with a β-D-Gal*p*A-(1→3)-α-D-Gal*p*NAc disaccharide or a longer terminal fragment (Fig. 1).

Sharing the same O-factor "a" seems to be also responsible for the cross-reactivity of *Proteus* genomospecies 4 LPS. (Fig. 1). However, there is an antibody fraction that recognizes the LPSs of *P. mirabilis* TG 115, CCUG 10701, and 41/57 and of *P. vulgaris* 44/57, but not *Proteus* genomospecies 4 (Table 3). This finding indicates that the *P. mirabilis* and *P. vulgaris* LPSs have an additional major epitope "b" in common which is absent from the *Proteus* genomospecies 4 LPS. This epitope could be linked to a common fragment within the main OPS chains, e.g. an α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A--(1→3)-α-D-Glc*p*NAc trisaccharide fragment as shown in Fig. 1.

The observed antigenic relationship of the three *P. mirabilis* LPSs and *P. vulgaris* 44/57 LPS is of the a,b,c/a,b,d type. The particular O-factors "c" and "d" are evidently associated with different fragments in the interior repeating units, whose exact structures remain to be determined. Based on these data, we propose 1) to classify the strains *P. mirabilis* 41/57 (O23) and TG 115 (formerly unclassified) to the *Proteus* O23a,23b,23c subgroup, 2) to reclassify strain CCUG 10701 (OB) from the O74 serogroup [10] to the same *Proteus* O23a,23b,23c subgroup, and 3) to classify *P. vulgaris* 44/57 and the serologically identical strains *P. mirabilis* 42/57 and *P. vulgaris* 43/57 [12] to the *Proteus* O23a,23b,23d subgroup.

A weak cross-reactivity of O-antisera against *P. mirabilis* TG 115 and CCUG 10701 with the LPSs of *P. mirabilis* O3 and O26 and *P. penneri* O71 is, most likely,

due to the presence of antibodies against a minor epitope associated with an amide of D-GalA with L-lysine (GalA6Lys), which is present in the outer core region of *P. mirabilis* TG 115 and CCUG 10701 LPS [3]. This epitope is shared by the O-polysaccharides of *P. mirabilis* O3 [17] and O26 [14, 24] and by the outer core region of *P. penneri* O71 LPS [20]. This conclusion was strongly supported by the present serological data. Indeed, absorption of *P. mirabilis* TG 115 and CCUG 10701 O-antisera with either *P. mirabilis* O3 or *P. penneri* O71 LPS (Table 3) completely removed cross-reactive antibodies to both LPSs (i.e. antibodies against GalA6Lys). Furthermore, Western blot demonstrated that *P. mirabilis* TG 115 and CCUG 10701 O-antisera recognized the high-molecular-mass LPS species of *P. mirabilis* O3 and O26 and the low-molecular-mass LPS species of *P. penneri* O71 (Fig. 2, see also above).

While the absorption of *P. mirabilis* TG 115 and CCUG 10701 O-antisera with *P. mirabilis* O26 LPS completely removed cross-reactive antibodies against GalA6Lys, the reactivity with *P. mirabilis* O26 LPS was only decreased after absorption with either *P. mirabilis* O3 or *P. penneri* O71 LPS. Most probably, the remaining cross-reactive antibodies recognize another LPS core epitope shared by the *P. mirabilis* O26 and *Proteus* O23 strains.

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