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Structures and serology of the O-antigens of *Proteus* strains classified into serogroup O17 and former serogroup O35

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Abstract

Introduction: Bacteria of the genus *Proteus* are facultative pathogens which commonly cause urinary tract infections. Based on the serological specificity of the O-chain polysaccharide of the lipopolysaccharide (O-polysaccharide, O-antigen), strains of *P. mirabilis* and *P. vulgaris* have been classified into 60 serogroups. Studies on the chemical structure and serological specificity of the O-antigens aim at the elucidation of the molecular basis and improvement of the serological classification of these bacteria.
Materials and Methods: The O-polysaccharide was prepared by acetic acid degradation of the lipopolysaccharide isolated from dried bacterial mass of each strain by hot phenol/water extraction. ^{1}H- and ^{13}C-NMR spectroscopy was used for structural studies. Serological studies were performed with rabbit O-antisera using enzyme immunosorbent assay, passive hemolysis test, and the inhibition of reactions in these assays as well DOC-PAGE and Western blot.
Results: Four *Proteus* strains belonging to serogroups O17 and O35 were found to possess similar O-polysaccharide structures, in particular having the same carbohydrate backbone built up of tetrasaccharide repeating units. However, they differ in the presence or absence of additional substituents, such as phosphoethanolamine in *P. mirabilis* O17 and glucose in *P. penneri* O17, as well as in the pattern and degree of O-acetylation of various monosaccharide residues. Serological studies also showed close relationships between the O-antigens studied.
Conclusions: Based on these data it is proposed to reclassify strain *P. mirabilis* PrK 61/57, formerly representing the O35 serogroup, into the serogroup O17 in the Kauffman-Perch classification system of *Proteus*.

Key words: *Proteus vulgaris*, *Proteus mirabilis*, O-antigen, O-polysaccharide structure, lipopolysaccharide, serological cross-reactivity.

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INTRODUCTION

Bacteria of the genus *Proteus* are facultative pathogens which commonly cause urinary tract infections that can lead to severe complications, such as the formation of kidney and bladder stones and acute or chronic pyelonephritis. The bacteria frequently cause infections in patients with a urinary catheter in place or after surgical intervention in the urogenital system [13]. Currently, *Proteus* bacilli are subdivided into five species, four of which, *P. mirabilis*, *P. vulgaris*, *P. penneri*, and *P. hauseri*, are of clinical importance [9]. Based on the serological specificity of the O-chain polysaccharide of the lipopolysaccharide (O-polysaccharide, O-antigen), strains of *P. mirabilis* and *P. vulgaris* have been classified into 60 serogroups [6, 8, 10]. Recently, a number of new serogroups were proposed for unclassified strains of these two genus as well as for *P. penneri* strains [12, 20 and references cited therein].

Serogroup O17 contains strains of all three species. The structures of the O-antigens of two of them,

P. mirabilis PrK 32/57 [19]* and *P. penneri* 16 [15], were reported by us earlier. Based on their structure similarity and serological relatedness, it was suggested to classify *P. penneri* 16 and a number of closely related *P. penneri* strains into the *Proteus* serogroup O17 [14]. In the present paper we report on the structures of the O-polysaccharides of *P. vulgaris* O17, strain PrK 33/57, and *P. mirabilis* O35, strain PrK 61/57, and on the serological cross-reactivity of *Proteus* O17-antisera. Based on the data obtained it is suggested to combine the O17 and O35 serogroups into one serogroup, O17.

MATERIALS AND METHODS

Bacterial strains

P. vulgaris O17, strain CCUG 4652 (PrK 33/57), came from the Culture Collection of the University of Goeteborg (Goeteborg, Sweden). *P. mirabilis* O17, strain PrK 32/57, and *P. mirabilis* O35, strain PrK 61/57, were from the Czech National Collection of Type Culture, Institute of Epidemiology and Microbiology (Prague, Czech Republic). *P. penneri* 16 (serogroup O17) came from the culture collection of the Institute of Microbiology and Immunology, University of Łódź (Poland).

Cultivation of bacteria and isolation of the lipopolysaccharide

Bacteria were cultivated under aerobic condition in nutrient broth (BTL, Łódź, Poland) with 1% glucose. The lipopolysaccharide (LPS) was isolated from dried bacterial mass of all strains by hot phenol/water extraction [16] and purified by treatment with a cold aqueous 50% solution of trichloroacetic acid followed by separating the precipitate by centrifugation and dialysis of the supernatant.

Mild acid degradation and O-deacylation

Acid degradation of the LPS was performed with aqueous 2% acetic acid (100°C, 2.5 h), and a lipid precipitate was removed by centrifugation (13,000×g, 20 min). The O-polysaccharide was isolated by gel-permeation chromatography on a column (2.6×60 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring using a Knauer differential refractometer. O-deacetylation of the O-polysaccharides was performed with 12% aqueous ammonia (37°C, 24 h).

For serological studies the LPS was saponified with 0.25 M NaOMe in absolute methanol (37°C, 15 h) to give alkali-treated LPS, which was isolated by precipitation with ethanol.

* *P. mirabilis* O17 strain PrK 32/57 studied earlier [19] was named erroneously as a *P. vulgaris* O17 strain.

NMR spectroscopy

^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker DRX-500 spectrometer equipped with an SGI INDY computer workstation using XWINNMR 2.6 program (Bruker) to acquire and process the NMR data. Samples were deuterium-exchanged by freeze-drying two times from D_2O and then examined as solutions in 99.96% D_2O at 30 or 60°C using internal acetone (δ_H 2.225, δ_c 31.45) as reference. A mixing time of 100 and 200 ms was used in 2-dimensional TOCSY and ROESY experiments, respectively. The other parameters used in the 2-dimensional experiments were essentially the same as described previously [4].

Serological techniques

Antisera were obtained by immunization of New Zealand white rabbits with heat-killed bacteria (100°C, 2.5 h) as described previously [1]. Enzyme immunosorbent assay (EIA), passive hemolysis test (PHT), and inhibition of passive hemolysis and EIA were performed as reported earlier [17]. DOC-PAGE and Western blot were carried out as described earlier [2].

RESULTS AND DISCUSSION

Structural studies of the O-polysaccharides of P. vulgaris O17 and P. mirabilis O35

NMR investigation of the O-polysaccharides derived from the LPSs of *P. vulgaris* O17 and *P. mirabilis* O35 revealed their marked structural similarity. The ^{1}H- and ^{13}C-NMR spectra of both O-polysaccharides contained signals for O-acetyl groups in non-stoichiometric amounts (δ_C 21.4-21.7, δ_H 2.10-2.18). In order to facilitate the assignment of the NMR signals, the O-polysaccharides were O-deacetylated with aqueous ammonia. The spectra of the O-deacetylated polysaccharides were essentially identical and indicated a tetrasaccharide repeating unit. In the ^{13}C-NMR spectrum there were signals for four anomeric carbons at δ 96.8, 99.3, 104.0, and 104.9, two nitrogen-bearing carbons at δ 52.9 and 53.1, one unsubstituted (δ 62.0) and one substituted (δ 69.5) $HOCH_2$-group, one CH_3-C group of a 6-deoxyhexose at δ 16.4, one N-acetyl, and one N-(3-hydroxybutyryl) group (CH_3 at δ 23.3 and 23.5, CH_2 at δ 45.8, CHOH at δ 65.8, CO at δ 175.0 and 175.2). Accordingly, the ^{1}H-NMR spectrum of the polysaccharides contained, *inter alia*, signals for four anomeric protons at δ 4.44-5.47, one CH_3-C group of a 6-deoxyhexose at δ 1.18, one N-acetyl group at δ 1.98 (6H), and one N-(3--hydroxybutyryl) group at δ 1.22 (CH_3), 2.38 and 2.44 (CH_2). The assignment of the spectra shown in Table 1 was performed using two-dimensional NMR techniques [3] and showed their virtual identity to the dephosphorylated polysaccharide of *P. mirabilis* O17 [19]. The latter consists of tetrasaccharide repeating units, containing

Table 1. ^{1}H- and ^{13}C-NMR data of the O-deacetylated polysaccharide of *P. vulgaris* O17 (chemical shifts, δ, ppm)

Residue	H1	H2	H3	H4	H5	H6
→4)-β-D-Glc*p*A-(1→	4.44	3.37	3.76	3.76	4.04	
→3)-α-D-Glc*p*NAc-(1→	5.47	4.12	3.62	3.54	3.64	3.76, 3.85
→2)-β-D-Fuc*p*3NHb-(1→	4.60	3.64	4.14	3.66	3.86	1.18
→6)-α-D-Glc*p*-(1→	5.47	3.49	3.60	3.24	3.72	3.64, 4.14
NHb		2.38, 2.44	4.18	1.22		
NAc		1.98				
	C1	C2	C3	C4	C5	C6
→4)-β-D-Glc*p*A-(1→	104.1	73.6	77.2	76.7	75.2	173.5
→3)-α-D-Glc*p*NAc-(1→	96.8	52.9	82.0	69.5	73.8	62.0
→2)-β-D-Fuc*p*3NHb-(1→	104.9	72.0	53.1	71.7	73.2	16.4
→6)-α-D-Glc*p*-(1→	99.3	72.5	73.3	71.1	71.5	69.5
NHb	175.0[a]	45.8	65.8	23.5[b]		
NAc	175.2[a]	23.3[b]				

[a, b]Assignment could be interchanged.

one residue each of D-glucose, N-acetyl-D-glucosamine (D-GlcNAc), D-galacturonic acid (D-GalA), and 3,6-dideoxy-3-[(*R*)-3-hydroxybutyryl]-D-galactose (D-Fuc3NHb), and has the structure shown in Fig. 1.

Proteus vulgaris O17 (PrK 33/57) [this work]

→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NHb-(1→6)-α-D-Glc*p*3Ac-(1→

Proteus mirabilis O17 (PrK 61/57; formerly *P. mirabilis* O35) [this work]

→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NHb4Ac-(1→6)-α-D-Glc*p*3Ac-(1→

Proteus mirabilis O17 (PrK 32/57)* [19]

Etn*P*
|
6
→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NHb-(1→6)-α-D-Glc*p*-(1→

Proteus penneri O17 (*P. penneri* 16 and related strains) [15]

α-D-Glc*p*
1
↓
2
→4)-β-D-Glc*p*A3Ac-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NHb-(1→6)-α-D-Glc*p*4Ac-(1→

Proteus mirabilis O16 [18]

Etn*P*
|
6
→6)-β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→2)-D-Rib-ol-5-*P*-(O→

Proteus vulgaris O37a,37b [17]

→4)-α-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc6Ac-(1→3)-β-D-Glc*p*A-(1→

Proteus vulgaris O37a,37ñ (former O46) [11,17]

→4)-α-D-Glc*p*6Ac-(1→3)-β-D-Glc*p*A4Ac-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Glc*p*A4Ac-(1→

Proteus penneri O19a,19b (*P. penneri* 31) [7]

Etn*P*
|
6
→4)-α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→

P. mirabilis O17 strain PrK 32/57 studied earlier [19] was named erroneously as a *P. vulgaris* O17 strain.

Fig. 1. Structures of the O-polysaccharides of *Proteus* strains. Abbreviations: D-Fuc*p*3NHb – 3,6-dideoxy-3-[(*R*)-3-hydroxybutyryl]-D-galactose, Etn*P* – phosphoethanolamine, D-Rib-ol-5-*P*, D-ribitol 5-phosphate.

A comparison of the NMR chemical shift data of the intact and O-deacetylated polysaccharides enabled determination of the location of the major O-acetyl group showing signals at δ_H 2.16 and δ_C 24.6. In the spectrum of the intact O-polysaccharide of *P. vulgaris* O17, the intensity of the Glc signals decreased and new signals for the corresponding O-acetylated Glc residue appeared. O-acetylation of the Glc residue at position 3 followed from a characteristic downfield shift of a part of the H3/C3 cross-peak from δ 3.60/73.3 in the ^{1}H,^{13}C HSQC spectrum of the O-deacetylated polysaccharide to δ 5.09/76.1 in the spectrum of the intact polysaccharide. In addition, it was confirmed by upfield displacements, caused by β-effects of O-acetylation [5], of parts of the C2 and C4 signals from δ 72.5 and 71.1 to δ 71.9 and 68.7, respectively. Based on the relative signal intensities it was concluded that approximately 50% of the Glc residues are 3-O-acetylated. No significant changes in the ^{13}C NMR chemical shifts could be observed for the other constituent monosaccharides, and the location of a minor O-acetyl group (δ_H 2.13 and δ_C 24.4, signal intensity ~0.15 related to that of the N-acetyl group) was not determined.

A similar splitting pattern of the Glc signals was observed in the spectra of the intact O-polysaccharide of *P. mirabilis* O35, including the distribution of the H3/C3 cross-peak between δ 3.60/73.3 and 5.00/77.1, the C2 signal between δ 72.5 and 71.6, and the C4 signal between δ 71.1 and 68.8. The degree of O-acetylation of the Glc residue was estimated as ~40%. In addition, the H4/C4 cross-peak of the Fuc3NHb residue in the ^{1}H,^{13}C HSQC spectrum shifted from δ 3.66/71.7 in the O-deacetylated O-polysaccharide to 5.05/75.5 in the intact polysaccharide and, correspondingly, the C3 signal shifted upfield from δ 53.9 to δ 52.6. These findings indicated the complete O-acetylation of the Fuc3NHb residue at position 4.

Based on the data obtained it was concluded that the O-polysaccharides of *P. vulgaris* O17 and *P. mirabilis* O35 have the structures shown in Fig. 1, which differ from each other only in the presence of an additional

O-acetyl group on the Fuc3NHb residue in the latter polysaccharide. Having the same main chain, they also resemble the O-polysaccharides of *P. mirabilis* O17 [19] and *P. penneri* 16 [15], from which they differ in the absence of phosphoethanolamine or a lateral glucose residue, respectively, as well as in the O-acetylation pattern (Fig. 1).

Serological studies

O-antisera against *P. vulgaris* O17 and *P. mirabilis* O17 were tested in PHT and EIA with LPSs of *Proteus* strains belonging to the O17 and O35 serogroups as well as with some other *Proteus* LPSs with structurally similar O-polysaccharides. Anti-*P. mirabilis* O35 serum was tested only in EIA. In PHT and EIA, both anti-O17 sera reacted strongly with the homologous LPSs and almost identically with the other O17 LPSs (Table 2). LPS of *P. mirabilis* O35 reacted strongly with the homologous antiserum and slightly more weakly with anti-O17 sera in EIA. Anti-*P. mirabilis* O35 serum showed a strong cross-reactivity with all *Proteus* O17 LPSs (Table 2).

All *Proteus* O17 LPSs as well as the corresponding isolated O-polysaccharides strongly inhibited the reactivity of anti-*P. vulgaris* O17 serum and anti-*P. mirabilis* O17 serum with the homologous LPS in both PHT and EIA (Table 3). The reactivity of anti-*P. mirabilis* O35 serum with the homologous LPS in EIA was strongly inhibited by the homologous LPS and O-polysaccharide, whereas a weaker inhibition was achieved with the *P. mirabilis* O17 antigens and almost no inhibition with those of *P. vulgaris* O17. Both the LPSs and the O-polysaccharide of *P. mirabilis* O35 were good inhibitors of the reaction of anti-*P. vulgaris* O17 serum with the homologous LPS in EIA, but the O35 LPS was much less active and the O35 O-polysaccharide inactive in the anti-*P. mirabilis* O17 serum/*P. mirabilis* O17 LPS system (Table 3).

The PHT and EIA results were essentially confirmed by Western blot data (Fig. 2). Anti-*P. vulgaris*

Table 2. Reactivity of the *Proteus* alkali-treated LPS (LPSOH) or LPS with anti-*P. vulgaris* O17 and anti-*P. mirabilis* O17 sera in PHT and EIA, respectively (reciprocal titer)

LPS or LPSOH from	PHT		EIA		
	P. mirabilis O17 antiserum	*P. vulgaris* O17 antiserum	*P. mirabilis* O17 antiserum	*P. vulgaris* O17 antiserum	*P. mirabilis* O35 antiserum
P. vulgaris O17	12 800	**12 800**	256 000	**512 000**	256 000
P. mirabilis O17	**25 600**	12 800	**512 000**	256 000	256 000
P. mirabilis O35	nd	nd	128 000	256 000	**1 024 000**
P. penneri 16 (O17)	25 600	12 800	256 000	512 000	512 000
P. vulgaris O37a,37b	3 200	1 600	32 000	2 000	32 000
P. vulgaris O37a,37c	6 400	12 800	32 000	128 000	2 000
P. mirabilis O16	800	<100	1 000	<1 000	<1 000
P. penneri 31	800	800	1 000	<1 000	nd

nd – not determined. Data of the homologous LPS or LPSOH are shown in bold type.

Table 3. Inhibition of the reactivity of anti-*P. vulgaris* O17 and anti-*P. mirabilis* O17 and O35 sera with the homologous alkali-treated LPS (LPSOH) or LPS in PHT and EIA, respectively (minimal inhibitory dose in ng)

Inhibitor (ng)	Inhibition in PHT		Inhibition in EIA		
	P. mirabilis O17	*P. vulgaris* O17	*P. vulgaris* O17	*P. mirabilis* O17	*P. mirabilis* O35
P. vulgaris O17 LPSOH	4.8	**2.4**	**2.4**	39.1	nd
P. vulgaris O17 LPS	4.8	**2.4**	**2.4**	19.5	5000
P. vulgaris O17 OPS	nd	nd	**2.4**	39.1	>5000
P. mirabilis O17 LPSOH	**2.4**	9.8	4.8	**4.8**	nd
P. mirabilis O17 LPS	**2.4**	9.8	4.8	**2.4**	312.5
P. mirabilis O17 OPS	nd	nd	4.8	**2.4**	312.5
P. mirabilis O35 LPS	nd	nd	4.8	625	**4.8**
P. mirabilis O35 OPS	nd	nd	9.8	>5000	**9.8**
P. penneri 16 (O17) LPSOH	2.4	4.8	4.8	19.5	nd
P. penneri 16 (O17) LPS	2.4	19.5	9.8	19.5	>5000
P. penneri 16 (O17) OPS	nd	nd	19.5	19.5	>5000
P. vulgaris O37a,37c LPSOH	>5000	2500	1250	<5000	nd
P. vulgaris O37a,37c LPS	>5000	78.1	78.1	<5000	>5000
P. vulgaris O37a, 37b LPSOH	>5000	>5000	>5000	>5000	nd
P. vulgaris O37a, 37b LPS	>5000	>5000	>5000	>5000	>5000

OPS – O-polysaccharide, nd – not determined. Data of the homologous LPS, LPSOH or OPS are shown in bold.

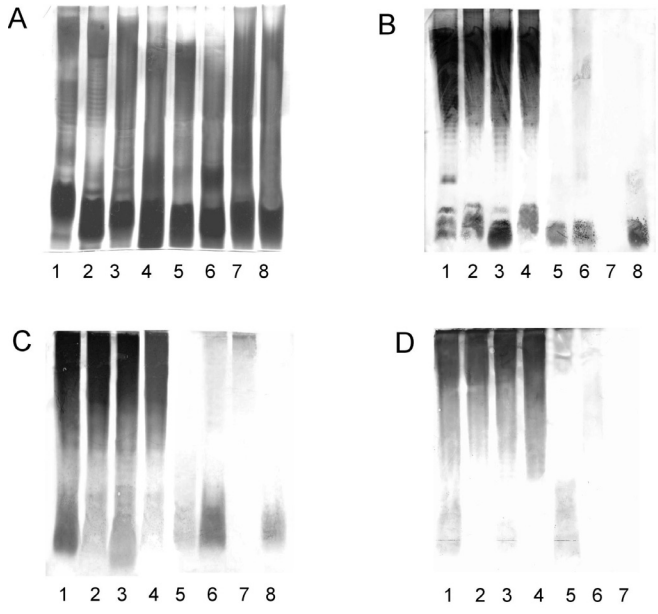

Fig. 2. Silver-stained sodium deoxycholate-PAGE (**A**) and Western blot of the LPS of *P. vulgaris* O17 (1), *P. mirabilis* O17 (2), *P. penneri* 16 (O17) (3), *P. mirabilis* O35 (4), *P. vulgaris* O37a,37b (5), *P. vulgaris* O37a,37c (6), *P. mirabilis* O16 (7), and *P. penneri* 31 (O19a,19b) (8) with anti-*P. mirabilis* O17 serum (**B**), anti-*P. vulgaris* O17 serum (**C**) and anti-*P. mirabilis* O35 serum (**D**).

O17 and anti-*P. mirabilis* O17 sera recognized both slowly and fast migrating bands of LPS of *P. vulgaris* O17, *P. mirabilis* O17 and O35, and *P. penneri* 16 (O17; Fig. 2B and C). These bands correspond to high-molecular-mass LPS species with a number of O-repeats and low-molecular-mass LPS species containing only the core-lipid A region, respectively. Anti-*P. mirabilis* O35 serum recognized slowly migrating bands of the homologous LPS and *P. mirabilis* O17 LPS and also reacted with both slowly and fast migrating bands of LPSs of *P. vulgaris* O17 and *P. penneri* 16 (O17; Fig. 2D).

These data demonstrate close serological relatedness of *Proteus* strains classified into the O17 and O35 serogroups and correlate with the similarity of their O--polysaccharide structures, which have the same carbohydrate main chain and differ in modifications with a terminal glucose residue, ethanolamine phosphate, and O-acetyl groups (Fig. 1). Previously it was suggested to divide the *Proteus* O17 serogroup into two subgroups, one for *P. mirabilis* PrK 32/57 and the other for *P. penneri* 16 (O17) and related strains [14]. The data obtained in this study show the expediency of further extension of the O17 serogroup to four subgroups by adding separate subgroups for *P. vulgaris* PrK 33/57 and *P. mirabilis* PrK 61/57, the latter being reclassified from the O35 serogroup.

The chemical basis for combining these strains into one serogroup is their identical backbone polysaccharide, which evidently carries the major O17 group epitope. Partial subgroup epitopes may be associated with ethanolamine phosphate (*P. mirabilis* PrK 32/57), the lateral glucose residue (*P. penneri* 16 and related strains), and *O*-acetyl groups, which are present in all strains except for *P. mirabilis* PrK 32/57. Minor antigenic factors may also reside in the LPS core region. For a more detailed characterization of the LPS epitopes, studies with cross-absorbed antisera are necessary.

O-antisera against *Proteus* O17 strains cross-reacted also with LPS of *P. vulgaris* O37 (Tables 2 and 3, Fig. 2), which most probably is due to the presence of a common β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc disaccharide in their O-polysaccharides. This finding is in accordance with the serological relationship of *P. mirabilis* O17 (PrK 32/57) and *P. vulgaris* O37 as revealed earlier using anti-*P. vulgaris* O37 sera [17]. Furthermore, anti-*P. mirabilis* O17 serum contains a minor fraction of antibodies cross-reacting with LPS of *P. mirabilis* O16, which most likely recognizes an epitope associated with the Etn*P*→6)-GlcNAc group shared by the O-polysaccharides of the two strains [18]. Finally, cross-reactivity of anti-*Proteus* O17 sera with fast migrating bands of LPSs of *P. vulgaris* O37a,37b and *P. penneri* 31 (O19a,19b [7]) in Western blot (Fig. 2) suggested the presence of a common epitope(s) in the LPS core region of these strains.

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