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Serological characterization of the O-specific polysaccharide of *Providencia alcalifaciens* O23

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature search

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Summary

Introduction: The genus *Providencia* belongs to the *Enterobacteriaceae* family and currently consists of five species: *P. alcalifaciens*, *P. heimbachae*, *P. rettgerii*, *P. rustigianii* and *P. stuartii*. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 63 O-serogroups and 30 H-serogroups. The O-antigenic specificity is defined by the structure of the O-antigen (O-specific polysaccharide – OPS), a part of the lipopolysaccharide (LPS, endotoxin), one of the major components of the outer membrane of Gram-negative bacteria and an important virulence factor of these bacteria. Among the bacteria of the *Enterobacteriaceae* family, the genus *Providencia* is one of the least studied in respect to its LPS structure and antigenic specificity. Studies of the chemical structures and the serological specificity of the O-antigens aim at the elucidation of the molecular basis of the serological classification of *Providencia* sp.

Materials and Methods: LPS and alkali-treated LPS of *P. alcalifaciens* O23 and serologically related *P. rustigianii* O14, *P. mirabilis* O13 and *P. myxofaciens* as well as O-antiserum against *P. alcalifaciens* O23 were used. Serological characterization of *P. alcalifaciens* O23 O-specific polysaccharide was done by use enzyme immunosorbent assay (EIA), passive hemolysis test (PHT) as well as by inhibition and sodium deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) of LPS and Western blot.

Results and Conclusions: The OPS of *P. alcalifaciens*, O23, contains an *N*-(D-glucuronoyl)-*N*-[(*R*)-1-carboxyethyl]-L-lysine residue (GlcAAlaLys). The LPS of *P. alcalifaciens*, O23, and other LPSs containing AlaLys from *Providencia* and *Proteus* strains were tested with rabbit anti-*P. alcalifiaciens* O23 serum. The serological data showed that a GlcAAlaLys-associated epitope plays a role as an antigenic determinant in the *P. alcalifaciens* O23 OPS and revealed the particular importance of glucuronic acid and the carboxyethyl group for the binding of O23-specific antibodies.

Key words: ***Providencia alcalifaciens*** **• lipopolysaccharide • O-antigen • O-serogroups •** ***N*$^{\varepsilon}$-[(*R*)-1-carboxyethyl]-L-lysine**

Abbreviations: ***2S,8S*-AlaLys** – N$^{\varepsilon}$-[(S)-1-carboxyethyl)]-l-lysine{"alanino-lysine"}, **EIA** – enzyme immunosorbent assay, **GlcA** – glucuronic acid, **LPS** –lipopolysaccharide, **LPSOH** – alkali-treated lipopolysaccharide, **OPS** – O-specific polysaccharide, **PHT** – passive hemolysis test

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INTRODUCTION

The genus *Providencia* belongs to the *Enterobacteriaceae* family and currently consists of five species: *P. alcalifaciens*, *P. heimbachae*, *P. rettgerii*, *P. rustigianii* and *P. stuartii*. They are facultative bacterial pathogens which cause intestinal as well as urinary tract infections[16]. Bacteria of these species are isolated from children with diarrhea, although they can also be found occasionally in non-diarrheic stools. *P. alcalifaciens* is more frequently isolated from the stool of adults with diarrhea who had traveled abroad[2,9]. *P. alcalifaciens* is able to invade intestinal mucosa and other cell types, and this has been proposed as the main virulence factor of these bacteria[1].

Like other bacteria from the tribe of *Proteae,* which includes also the genera *Proteus* and *Morganella, Providencia* sp. have an O-antigen, a flagellar H-antigen and a capsular K-antigen, which form the basis for their serological classification[17]. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 63 O-serogroups and 30 H-serogroups[7,8,18]. These bacteria are serologically related to *Escherichia coli*, *Proteus, Morganella, Salmonella* and *Shigella*[22]. The O-antigenic specificity is defined by the structure of the O-antigen (O-specific polysaccharide – OPS), a part of the lipopolysaccharide (LPS, endotoxin), one of the major components of the outer membrane of Gram-negative bacteria. LPS is also considered as a important virulence factor of these bacteria. Among bacteria of the *Enterobacteriaceae* family, the genus *Providencia* is one of the least studied in respect to LPS structure and antigenic specificity. Studies of the chemical structures and serological specificity of the O-antigens aim at the elucidation of the molecular basis of the serological classification of *Providencia* sp. By now, structures of the O-specific polysaccharides of the LPS of *Providencia* O5[28], O7[5], O14[15], O16[14] O21[11] and O23[12, 13] have been established. The chemical structure of the OPS of *P. alcalifaciens*, O23, which contains an N^{ε}-[(*R*)-1-carboxyethyl]-N^{α}-(D-glucuronoyl)-L-lysine, is shown in Fig. 1. We shall now report on the serological properties of the *P. alcalifaciens* O23 O-antigen.

MATERIALS AND METHODS

Bacterial strains and LPS

P. alcalifaciens O23:H13 strain 738/49 came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest, Hungary). The cultivation of the bacteria and isolation of its LPS and OPS were performed as described previously[16]. The LPSs and OPSs of *Providencia rustigianii* O14[15], *Proteus mirabilis* O13[19,25], *P. mirabilis* O14[19] and *Proteus myxofaciens*[23] were obtained as reported earlier.

Preparation of alkali-treated LPS

Alkali-treated LPSs (LPSOH) were prepared by treatment of the LPS with 0.25 M NaOMe in absolute MeOH at 37°C for 15 h[27], after which the product was dissolved in water and lyophilized.

Serological techniques

O-antiserum against *P. alcalifaciens* O23 was prepared by immunization of New Zealand white rabbits with heat-killed bacteria (100°C, 2.5 h) as described previously[3]. An enzyme immunosorbent assay (EIA) and a passive hemolysis test (PHT) were performed as reported[26]. The LPS and alkali-treated LPS were used as antigens in EIA and PHT, respectively. Absorption of antiserum (1ml, diluted 1:50 with phosphate-buffered saline) was carried out at 4°C for 1 h with packed sheep red blood cells (100 μl) coated with the respective alkali-treated LPS (200 μg LPS in 0.2 ml sheep red blood cells). The level of antibodies after absorption was determined using PHT. Sodium deoxycholate polyacrylamide gel electrophoresis of the LPS and Western blot with anti-*P. alcalifaciens* O23 serum were performed as described previously[4].

RESULTS AND DISCUSSION

A peculiar feature of the O-antigen of *P. alcalifaciens* O23 is the presence of N^{ε}– (1–carboxyethyl)lysine (alani-

Table 1. Reactivity of anti-*P. alcalifiaciens* O23 serum with homologous and heterologous *Providencia* and *Proteus* LPS and LPSOH in EIA and PHT, respectively (reciprocal titer)

Antigen from	PHT	EIA
P. alcalifaciens O23	102 400	2 048 000
P. mirabilis O14 (29/57)	51 200	128 000
P. mirabilis O14 (EU313)	25 600	64 000
P. myxofaciens	51 200	32 000
P. rustigianii O14	3 200	4 000
P. mirabilis O13	3 200	4 000
P. vulgaris O44	200	< 1 000

Table 2. Reactivity with LPSOH or LPS from the homologous strain in PHT and EIA of anti-*P. alcalifaciens* O23 serum absorbed with LPSOH from *Proteus* and *Providencia* strains (reciprocal titer)

Absorption with	Reactivity in	
	PHT	EIA
Control	51,200	2,048,000
LPSOH of:		
P. alcalifaciens O23	200	1,000
P. mirabilis O14 (29/57)	12,800	1,024,000
P. mirabilis O14 EU313	12,800	512,000
P. myxofaciens	25,600	512,000
P. rustigianii O14	25,600	1,024,000
P. mirabilis O13	25,600	1,024,000

Sheep red blood cells were used as control.

nolysine – AlaLys), which amidates the carboxyl group of D-glucuronic acid[12, 13] (Fig. 1). AlaLys was also found in O-antigens of *P. rustigianii* O14[19], *P. mirabilis* O13[20, 25] and *P. myxofaciens*[23]. In *P. myxofaciens*, *2S,8R*-AlaLys amidates the D-glucuronic acid residue as in *P. alcalifaciens* O23 LPS, whereas O-antigens of *P. rustigianii* O14 and *P. mirabilis* O13 contain amides of D-GalA with *2S,8S*-AlaLys and *2S,8R*-AlaLys, respectively (Fig 1). Amides of D-glucuronic acid and D-galacturonic acid with amino acids, including L-lysine and L- or D-alanine, have been previously found in O-antigens of the genus *Proteus.* These components form epitopes important in the serological specificity of O-antigens[10]. Therefore, LPSs with known structures from *Providencia* and *Proteus* strains were used in serological studies and tested with rabbit polyclonal antiserum against *P. alcalifaciens* O23 in PHT and EIA (Table 1, Fig. 2), as well as in Western blot (Fig. 3). In addition, absorption and inhibition techniques were employed (Tables 2 and 3).

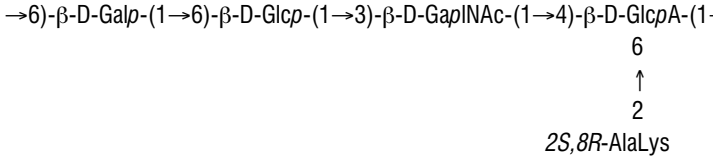
Strong reactions of anti-*P. alcalifaciens* O23 serum in both assays were observed in the homologous system (Table 1). In heterologous systems, significant cross-reactions of this serum were demonstrated with the LPS and LPSOH of *P. mirabilis* O14 and *P. myxofaciens.* Weaker cross-reactivity of anti-*P. alcalifaciens* O23 serum was observed with the LPSOH of *P. rustigianii* O14, as well as with *P. mirabilis* O13 in PHT, whereas these antigens were practically not reactive in EIA (titers of cross-reactions and reactions in the homologous system were 4,000 and 2,048,000, respectively; Table 1). A very weak reactivity was also noticed with the LPSOH of *P. vulgaris* O44. No reaction of anti-*P. alcalifaciens* O23 serum in both serological assays was observed with the LPSs of *P. mirabilis* O3, O26, O27, O28 and O29, as well as with *P. penneri* 1, 14, and 34 (data not shown). Differences in reactions of anti-*P. alcalifaciens* O23 serum with *P. rustiganii* O14 and *P. mirabilis* O13 in the passive hemolysis test in comparison with the reactivity

Table 3. Results of inhibition of PHT and EIA in the antigen-antibody system of *P.alcalifaciens* O23 LPSOH or LPS – homologous antiserum

Inhibitor	Inhibitory dose (ng) in	
	PHT	EIA
LPS *P. alcalifaciens* O23	1.0	< 2.5
PS *P. alcalifaciens* O23	< 1.0	2.5
LPS *P. mirabilis* O14 (29/57)	> 10,000	10,000
LPS *P. mirabilis* O14 EU313	> 10,000	> 10,000
LPS *P. myxofaciens*	> 10,000	> 10,000
LPS *P. rustigianii* O14	> 10,000	> 10,000
LPS *P. mirabilis* O13	> 10,000	> 10,000
L-Ala-β-D-GlcA-PAA	> 50,000	12,000

PAA – polyacrylomide

A



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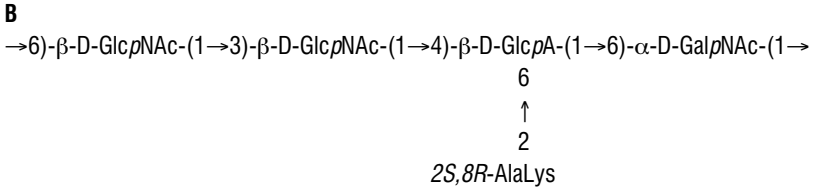

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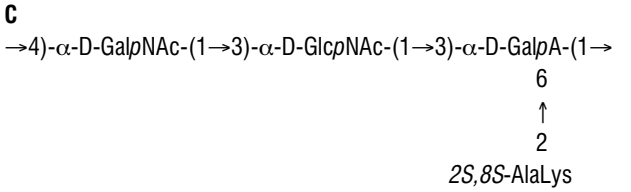

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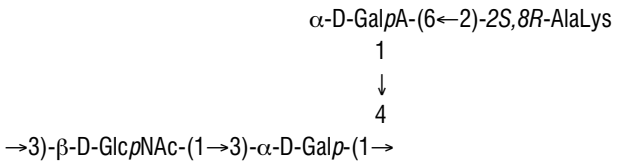


Figure1. Structures of the O-specific parts of the LPSs of *Providencia alcalifaciens* O23 (**A**)[13], *Proteus myxofaciens* (**B**)[23], *Providencia rustigianii* O14 (**C**)[15], and *Proteus mirabilis* O13 (**D**)[20] containing amides of D-glucuronic acid with N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine (*2S,8R*-AlaLys; **A, B**) and D-galacturonic acid with N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine (*2S,8S*-AlaLys; **C**) and N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine (*2S,8R*-AlaLys; **D**).

of this antiserum in the immunosorbent assay can be accounted for by the different exposure of the carbohydrate epitope in LPS depending on the antigen carrier used in the serological assays, sheep red blood cells, and the plastic surface, respectively. The differences in binding capacity of antibodies against *P. alcalifaciens* O23 with the homologous LPS and heterologous LPSs of *P. mirabilis* O13 and O14, as well as *P. myxofaciens* and *P. rustigianii* O14 in EIA are shown in Fig. 2.

The reactivity of anti-*P. alcalifaciens* O23 serum in EIA and PHT was completely abolished when the antiserum was absorbed with the homologous LPS. Absorption with the heterologous LPSs influenced the antiserum titer only insignificantly (Table 2).

In Western blot, anti-*P. alcalifaciens* O23 serum recognized both slow and fast migrating bands of the homologous LPS, which correspond to high- and low-molecular-mass LPS species with or without the O-specific chain, respectively. It also reacted with the high-molecular-mass LPSs of *P. rustigianii* O14, *P. mirabilis* O13 and O14 as well as *P. myxofaciens* (Fig. 3), thus indicating that a cross-reactive epitope(s) resides on the O-specific part of these LPS. The cross-reactivity of anti-*P. alcalifaciens* O23 serum with the bacterial cell suspension of the *P. rustigianii* O14 strain was observed earlier when the serological identification scheme of *Providencia* was developing[18].

The specificities of the cross-reactions observed were checked with the inhibition of passive hemolysis test and the immunosorbent assay with the LPSs, the polysaccharide chain (PS) of LPS, and the synthetic antigens acrylamide-based glycoconjugates containing alanine or amides of β-D-GlcA and β-D-GalA with alanine and lysine residues[6] (Table 3). It was found that as little as 1.0 or 2.5 ng of the LPS or PS of *P. alcalifaciens* O23 was sufficient to inhibit the reaction with homologous antiserum in PHT and EIA, respectively. The other LPS or PS showed much lower inhibitory activity or were inactive up to a dose 5,000 ng, which is in agreement with results obtained in PHT and EIA. From the synthetic antigens, L-Ala-β-D-GlcA-PAA showed inhibition, but only in high dose of 12.5 μg (Table 3).

Interpretation of the serological data is possible when they are compared with the structure of the repeating units of the LPSs used in the study (Figs. 1 and 4). The structures of the O-antigens used in this study have been established. The O-specific parts of heterologous LPSs contain sugars in common with the O-antigen of

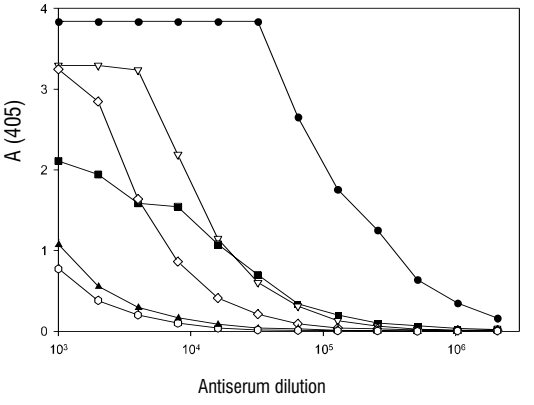


Figure 2. EIA binding curves of anti-*Providencia alcalifaciens* O23 serum to LPSs of the following strains: ● *P. alcalifaciens* O23; ▽ *P. mirabilis* O14 (29/57); ◊, *P. mirabilis* O14 (EU 313); ■, *P. myxofaciens*; ▲, *P. rustigianii* O14; ◯, *P. mirabilis* O13 in ELISA.

P. alcalifaciens O23. The lack of cross-reactivity of *P. alcalifaciens* O23 antiserum with the LPSs of *P. mirabilis* O3 and O29, as well as with *P. penneri* 1 and 34 (data not shown), indicated the unimportance of the following epitopes for their serological specificity: β-D-GalNAc(1→4)-β-GlcA, α-D-Gal-(1→6)-β-D-Glc-(1→3)-β-D-GalNAc and β-D-Glc-(1→3)-β-D-GalNAc, which are present in the main chain of the homologous O-antigen. The negative reaction of *P. alcalifaciens* O23 antiserum with O-antigens from *P. mirabilis* O3, O26, O27 and O28, which contain amides of galacturonic or glucuronic acid with lysine, showed that this amino acid alone or as a part of an amide cannot bind antibodies present in *P. alcalifaciens* O23 antiserum. Therefore, the results of the serological study suggest that the *P. alcalifaciens* O23 serogroup-specific antigenic determinant most probably shares *N*-(D-glucuronoyl)-*N*-[(*R*)-1-carboxyethyl)-L-lysine (Fig. 4). Indeed, a particularly strong reactivity of *P. alcalifaciens* O23 antiserum with *P. myxofaciens* O-antigen, which contains this epitope, was noticed. A weak reaction of *P. alcalifaciens* O23 antiserum with *P. rustigianii* O14 and *P. mirabilis* O13, LPSs that contain GalA(AlaLys), suggest the importance of the β-glucuronic acid residue in the epitope GlcA(AlaLys) present in *P. alcalifaciens* O23 LPS. The strong cross-reaction of *P. alcalifaciens* O23 antiserum with the O-antigen of *P. mirabilis* O14 could be explained by some similarity of this O-antigen with the O-specific part of *P. alcalifaciens* O23 LPS. Both O-antigens are built up of repeating units containing four sugar residues in the main chain substituted with Ala-Etn*P* and AlaLys in *P. mirabilis* O14 and *P. alcalifaciens* O23 LPS, respectively (Figs. 1 and 4). Thus, it can be concluded that the carboxyethyl group is important for the specificity of the O-antigen of *P. alcalifaciens* O23. Most likely, an alanine residue that is linked to the GlcA residue in the O-antigen of *P. vulgaris* O44 is responsible for the weak cross reactivity with anti-*P. alcalifaciens* O23 serum. The weak inhibition with L-Ala-β-D-GlcA-PAA (Table 3) also suggests the importance of the Ala residue in GlcA(AlaLys) epitope for manifesting the specificity of *P. alcalifaciens* O23 O-antigen.

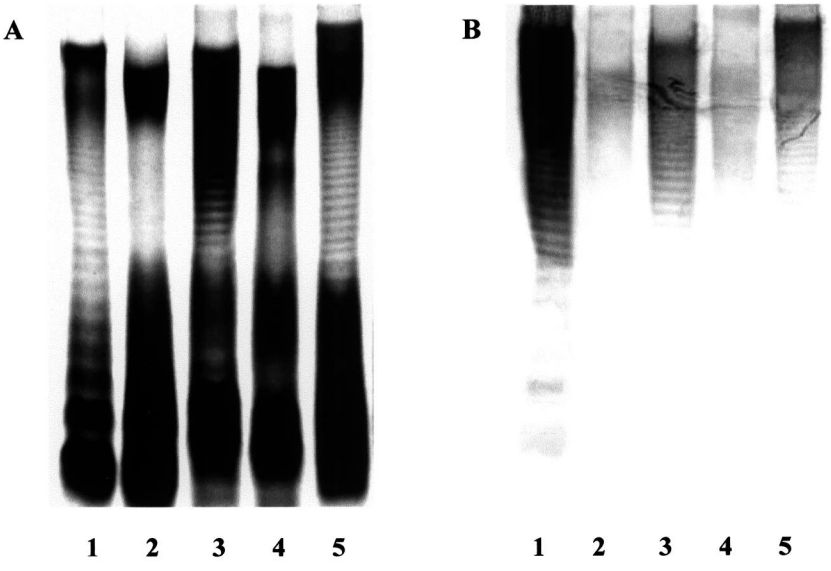
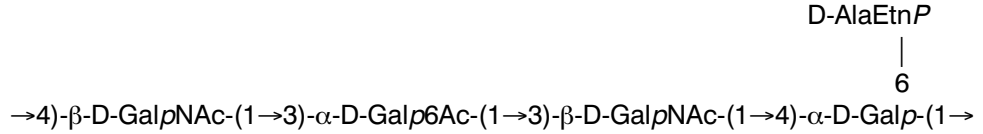


Figure 3. Sodium deoxycholate polyacrylamide gel electrophoresis (**A**) and Western blot with anti-*P. alcalifaciens* O23 serum (**B**) of the LPS of *P. alcalifaciens* O23 (lane 1), *P. rustigianii* O14 (lane 2), *P. myxofaciens* (lane 3), *P. mirabilis* O13 (lane 4), *P. mirabilis* O14 (EU3135; lane 5).

A



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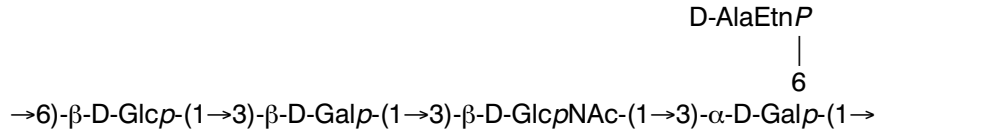

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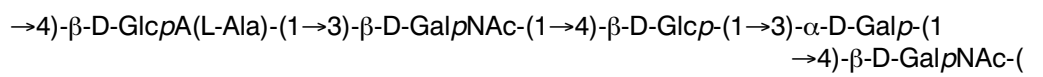

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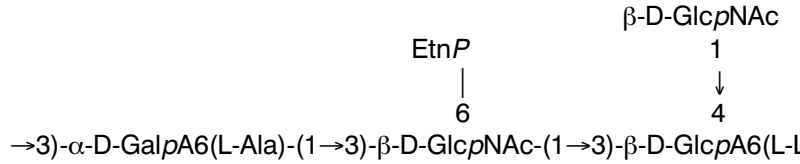

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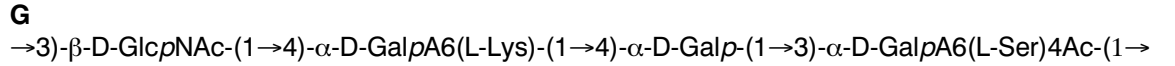

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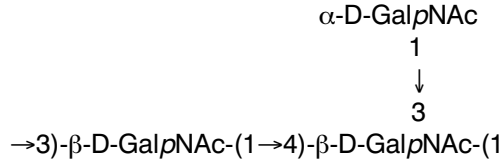

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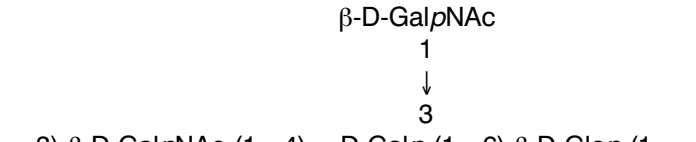

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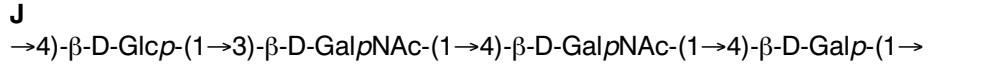

Figure 4. Structures of the O-specific parts of the LPSs of *P.mirabilis* O14 29/57 (**A**)[20], *P.mirabilis* O14 (EU3135; **B**)[20], *P.vulgaris* O44 (**C**)[21], *P.mirabilis* O3 (**D**)[10], *P.mirabilis* O26 (**E**)[10], *P.mirabilis* O27 (**F**)[10], *P.mirabilis* O28 (**G**)[10], *P.mirabilis* O29 (**H**)[10], *P.penneri* 1 (**I**)[24], *P.penneri* 34 (**J**)[10].

The results of our studies of the serological specificity of *P. alcalifaciens* O23 LPS reported in this paper, as well as previous findings[10, 15, 20, 21, 23], show that, together with the major epitope within LPS, which defines the group specificity of the *Providencia* strains, there are numerous minor epitopes which provide a serological cross-reactivity of strains belonging to different serogroups and even a different genus of bacteria. This should be taken into account when creating a detailed serological classification scheme of bacteria.

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