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Structure of an abequose-containing O-polysaccharide from *Citrobacter freundii* O22 strain PCM 1555

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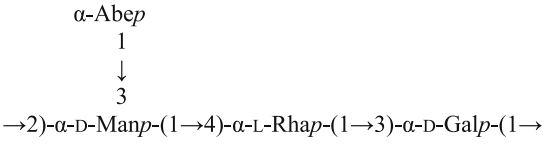
ABSTRACT

The lipopolysaccharide of *Citrobacter freundii* O22 (strain PCM 1555) was degraded under mild acidic conditions and the O-polysaccharide released was isolated by gel chromatography. Sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional $^{1}H,^{1}H$ ROESY and $^{1}H,^{13}C$ HMBC experiments, showed that the repeating unit of the O-polysaccharide has the following structure:



where Abe is abequose (3,6-dideoxy-D-*xylo*-hexose). SDS–PAGE and immunoblotting revealed that the O-antigen of *C. freundii* O22 is serologically indistinguishable from those of *Salmonella* group B serovars (Typhimurium, Brandenburg, Sandiego, Paratyphi B) but not related to other abequose-containing O-antigens tested (*Citrobacter werkmanii* O38 and *Salmonella* Kentucky) or colitose (L enantiomer of abequose)-containing O-antigen of *Escherichia coli* O111.

1. Introduction

The genus *Citrobacter* was designated first by Braak[1] as well as by Werkman and Gillen[2] for a group of Gram-negative citrate-utilizing and lactose-fermenting coli-form bacteria. Strains of *Citrobacter* are inhabitants of the intestinal tract and are found in sewage, surface waters and food contaminated with faecal material. These bacteria may cause gastroenteritis and opportunistic infections[3] as well as urinary and respiratory tract diseases, especially in immunocompromised hosts, which may be associated with meningitis, brain abscesses and neonatal sepsis.[3,4] These microorganisms were described under a variety of names, such as *Padlewskia, Levinea, Colobactrum, Paracolobactrum* and Bethesda-Ballerup group unless in 1958, the International Subcommittee of Taxonomy of *Enterobacteriaceae* adopted the term *Citrobacter freundii* for the group of organisms which comprised also *C. koserii* and *C. amalonaticus*.[3,5–8] At present, the genus *Citrobacter* contains 11 species and 43 O-serogroups,[6–11] the eight species being added to the genus on the basis of DNA relatedness and biochemical studies.[9,12,13]

Based on the sugar composition of the lipopolysaccharide (LPS), *Citrobacter* strains were classified into 20 chemotypes.[7] Eleven from them are identical to chemotypes that occur in *Salmonella* and *Escherichia coli,* and numerous serological cross-reactions between strains of the three genera have been observed. Since the serological specificity of Gram-negative bacteria is defined by the fine structure of the O-polysaccharide part of their LPS (O-antigen), detailed structural studies on *Citrobacter* O-polysaccharides have been carrying out in several laboratories with the aims to substantiate the serological cross-reactivity between *Citrobacter* and other genera on the molecular basis and to improve the existing classification of *Citrobacter* strains. More than 30 structures of O-antigens derived from serologically different *Citrobacter* strains have been elucidated so far.[11,14–17] Our immunochemical studies showed that *Citrobacter* O-antigens from some different serogroups are closely related and, on the contrary, O-antigens from the same serogroup may be antigenically diverse.

Now we report on the structure of the O-polysaccharide of *Citrobacter* PCM 1555, which, according to the revised classification

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scheme,[10] belongs to *C. freundii* and represents serogroup O22. The O-antigen of this bacterium was found to contain abequose (3,6-dideoxy-D-*xylo*-hexose) and to be closely related to the O-antigens of *Salmonella* serogroup B. Serological relatedness between the LPS of *Citrobacter* O22 and abequose-containing LPS of some other *Citrobacter* and *Salmonella* strains was investigated as well.

2. Experimental

2.1. Bacterial strains, isolation and degradation of the lipopolysaccharide

C. freundii O22:64 (PCM 1555; IHE Be 86/57; P.R. Edwards strain Ind 1219; GISK 120045[7,8,10]), *Citrobacter werkmanii* O38 (PCM 1489), *E. coli* O111 (PCM 277), *Salmonella* serovars Kentucky (PCM 1720), Typhimurium (PCM 999), Brandenburg (PCM 1734), Sandiego (PCM 1730) and Paratyphi B (PCM 855) were from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Bacteria were cultivated in a liquid medium with aeration at 37 °C for 24 h, harvested and freeze-dried. LPS was obtained in a 3.3% yield from dry bacterial mass of *C. freundii* O22 by phenol–water extraction[18] and was purified as described.[19] The LPS (300 mg) was degraded with aqueous 1% HOAc (30 ml, 100 °C, 90 min), and, after removal of a lipid A sediment, the carbohydrate-containing material (52% of the LPS mass) was fractionated by GPC on a column (2.0 × 100 cm) of Sephadex G-50 in 0.05 M aqueous pyridinium acetate buffer pH 5.6 to give O-polysaccharide (fraction P_1), an intermediate fraction (P_2), a core oligosaccharide (fraction P_3) and a low-molecular-mass material containing 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) in 5.0%, 2.1%, 66.7% and 26.2% yields, respectively, of the total amount of carbohydrate material eluted from the column.

2.2. Analytical procedures

Sugar and methylation analyses were carried out as described.[15] For sugar analysis the O-polysaccharide was hydrolysed with 2 M CF_3CO_2H (0.5 mg, 120 °C, 2 h) or 10 M HCl (80 °C, 30 min) or 0.1 M HCl (80 °C, 2 h). Sugars were converted conventionally into alditol acetates and analysed by GLC–MS[20] using a Hewlett–Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and a temperature programme from 150 to 270 °C at 8 °C min^{-1}. Methylation of the O-polysaccharide (0.6 mg) was performed according to the Gunnarsson[21] procedure, the methylated products were recovered by extraction with chloroform/water (1:1, v/v), hydrolysed with 2 M trifluoroacetic acid (120 °C, 2 h), and the partially methylated monosaccharides were converted into the alditol acetates and analysed by GLC–MS as above. O-polysaccharide was hydrolysed to monosaccharides with 0.1 M HCl (80 °C, 2 h) or 2 M CF_3CO_2H (120 °C, 2 h) and subjected to paper and thin-layer chromatography. Paper chromatography was performed on the Whatman 1 paper in a butanol/pyridine/water (v/v/v/ 4:3:1) solvent system and TLC was carried out on DC-Fertigplatten Kieselgel plates in a solvent system of EtOAc/pyridine/HOAc/water (v/v/v/v 5:5:1:3). The separated sugars were stained on the chromatograms with the $AgNO_3$/NaOH or molybdate/H_2SO_4 reagent (10 g $Ce(SO_4)_2$, 25 g $(NH_4)_2MoO_4$, 100 mL concd H_2SO_4, 900 mL H_2O; at 100 °C for 2–5 min), respectively. Galactose content was determined using D-galactose oxidase[22] after hydrolysis of the O-polysaccharide with 2 M trifluoroacetic acid (120 °C, 2 h).

2.3. Serological methods

Rabbit serum against the whole cells of *C. freundii* O22:64 (Be 86/57; PCM 1555) was prepared as reported earlier.[23] Anti-*E. coli* O111 serum was purchased from Biomed (Kraków). SDS–PAGE of LPS was performed by the method of Laemmli.[24] The gels were stained with the silver reagent.[25] Immunoblotting was carried out as described.[26] After separation in SDS–PAGE, the LPS were transblotted from the gel into an Immobilon P (Millipore) membrane, which was incubated with antiserum, washed with Tris-buffered saline (20 mM Tris–HCl, 50 mM NaCl, 0.05% Tween-20, pH 7) and incubated with alkaline phosphatase conjugated with goat anti-rabbit IgG. The immunoblot was visualised with the staining reagent (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.05 M Tris/HCl pH 9.5 containing 5 mM $MgCl_2$).

2.4. NMR spectroscopy

Prior to the measurements, the samples were freeze-dried twice from 99.9% ^2H_2O and dissolved in 99.96% ^2H_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance 600 MHz spectrometer at 30 °C; chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45) as reference. The NMR spectra were assigned by two-dimensional $^1H,^1H$ COSY, TOCSY, ROESY, H-detected $^1H,^{13}C$ HSQC and HMBC experiments, which were performed using standard Bruker software. A mixing time of 150 or 200 ms was used in TOCSY and NOESY experiments, respectively.

3. Results and discussion

Mild acid degradation of the LPS of *C. freundii* PCM 1555 afforded the O-polysaccharide (fraction P_1), which was separated from a low-molecular-mass core oligosaccharide (fraction P_3) on Sephadex G-50 column. Thin-layer and paper chromatography (0.5 mg O-polysaccharide hydrolysed with 0.1 M HCl, 80 °C, 2 h) revealed the presence of Gal, Man, Rha and another sugar with the mobility R_{Rha} = 1.14 identical to colitose (3,6-dideoxy-L-*xylo*-hexose) derived from the hydrolysate of *E. coli* O111 LPS. Sugar analysis of the O-polysaccharide by GLC–MS of the acetylated alditols revealed Rha, Man, Gal and a 3,6-dideoxyhexose (fragment ion peaks at *m/z* 69, 83, 96, 103, 129, 143, 156, 231) with the same retention time as colitose and its D enantiomer (abequose) in molar ratios 0.5:0.7:1.0:0.4 (Table 1). Determination of the absolute configuration by GLC of the acetylated (*S*)-2-octyl glycosides[27] showed that Man and Gal have the D configuration, whereas Rha has the L configuration. The 3,6-dideoxyhexose was found to have the D configuration and hence, is abequose (Abe). The configuration of galactose (content 22.5%) was also confirmed by enzymatic test with D-galactose oxidase[22] and those of the other sugars were confirmed by NMR data (see below).

Methylation analysis of the O-polysaccharide (hydrolysis with 10 M HCl, 80 °C, 30 min) revealed methylated derivatives of 4-substituted rhamnose, 3-substituted galactopyranose and 2,3-disubstituted mannopyranose in the ratios 0.8:1.2:1.0, respectively. A low amount of a terminal 3,6-dideoxyhexose derivative (*m/z* 69, 75, 83, 101, 117, 131, 143, 157) was also detected, when 0.1 M HCl (80 °C, 2 h) was used for hydrolysis.

Table 1
GLC–MS sugar analysis data of the O-polysaccharide of *Citrobacter* O22 after hydrolysis using different acids

Monosaccharide	T_R[a]	Molar content related to galactose		
		2 M CF_3CO_2H	0.1 M HCl	10 M HCl
Abe	0.53	0.2	0.8	0.0
Rha	0.67	0.7	0.3	0.3
Man	0.99	0.9	0.4	0.7
Gal	1.01	1.0	1.0	1.0

[a] GLC retention time related to peracetylated glucitol.

The ^{13}C NMR spectrum of polysaccharide (Fig. 1) demonstrated its regular structure. It contained signals for four sugar residues, including those for four anomeric carbons at δ 101.1–103.3, two unsubstituted CH_2OH groups (C-6 of Man and Gal) at δ 62.0 and 62.5, two CH_3 groups at δ 17.0 and 18.6 (C-6 of Rha and Abe), one C–CH_2–C group (C-3 of Abe) at δ 34.4 and 15 oxygen-bearing sugar-ring carbons in the region δ 64.8–83.0. Accordingly, the ^1H NMR spectrum contained signals for four anomeric protons at δ 5.06–5.33, two CH_3 groups (H-6 of Rha and Abe) at δ 1.34 and 1.19, one C-CH_2-C group (H-3 of Abe) at δ 1.98 and 2.02 and sugar-ring protons in the region δ 3.56-4.12. As judged by the absence of non-anomeric carbon signals in a lower field than δ 83, all sugar residues are in pyranose form.[28]

The ^1H and ^{13}C NMR spectra of the polysaccharide were assigned using ^1H,^1H COSY, TOCSY, ROESY, ^1H,^{13}C HSQC, HSQC–TOCSY and HMBC experiments (Table 2). The spin system of Rha*p* was distinguished based on the correlations between Rha*p* H-6 (δ 1.34) and all the other protons of this residue in the TOCSY spectrum, whose signals were assigned using COSY. The assignment of Rha*p* ^{13}C signals was performed using the data of ^1H,^{13}C HSQC experiment and confirmed by ^1H,^{13}C HSQC–TOCSY, which revealed the correlations of Rha*p* H-2 with all carbons of the residue. The chemical shift δ 69.4 of Rha*p* C-5 indicated the α configuration of this residue.[29] A significant downfield displacement of Rha*p* C-4 signal from δ 73.5 in the non-substituted α-Rha*p* to δ 83.0 in the polysaccharide showed substitution at position 4.

In the ^1H NMR spectrum, the signals at δ 1.19 belonged evidently to H-6 and those at δ 1.98 and 2.02 to H-3 of Abe*p*. The signals for H-1, H-2 and H-4 of Abe*p* were assigned by correlations with H-3 and that for H-5 was assigned by a correlation with H-6 in the COSY and TOCSY spectra. The ^{13}C NMR signals of Abe*p* were assigned using a ^1H,^{13}C HSQC experiment and confirmed by ^1H,^{13}C HSQC–TOCSY showing H-1/C-2 and H-1/C-4 correlations and by a H-5/C-4 correlation in the ^1H,^{13}C HMBC spectrum. The ^{13}C NMR chemical shifts of Abe*p* appeared to be characteristic for terminal 3,6-dideoxy-α-*xylo*-hexopyranoside.[30]

Man*p* and Gal*p* residues were distinguished by relatively small $J_{2,3}$ ~3 Hz and large $J_{3,4}$ ~10 Hz for the former and relatively large $J_{2,3}$ ~10 Hz and small $J_{3,4}$ ~3 Hz for the latter. The ^1H and ^{13}C NMR signals for Man*p* were assigned using COSY, TOCSY and ^1H,^{13}C HSQC experiments with the aid of HMBC data. Particularly, the HMBC spectrum demonstrated *intra*-residue H-1/C-3, H-1/C-5,H-2/C-3

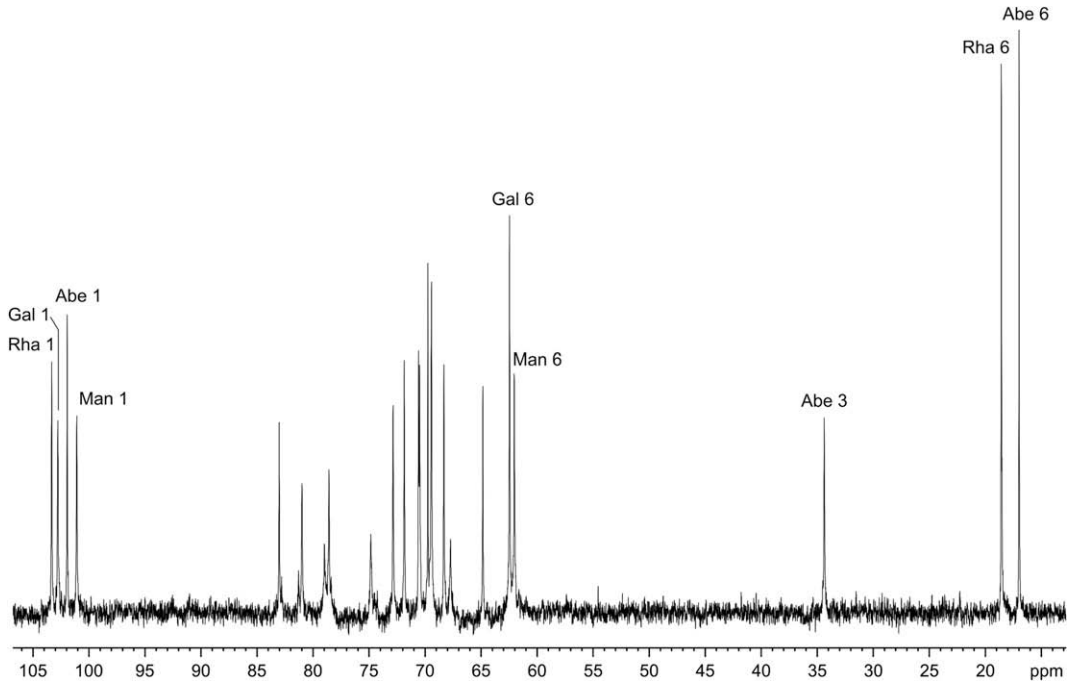

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *C. freundii* O22.

Table 2
^1H and ^{13}C NMR data (δ, ppm) of the O-polysaccharide of *Citrobacter* O22

Residue	C-1 *H-1*	C-2 *H-2*	C-3 *H-3* (*3a,3b*)	C-4 *H-4*	C-5 *H-5*	C-6 *H-6* (*6a,6b*)
→4)-α-L-Rha*p*	103.3 *5.06*	71.8 *4.08*	70.5 *3.98*	83.0 *3.56*	69.4 *3.94*	18.6 *1.34*
→3)-α-D-Gal*p*	102.8 *5.18*	69.4 *3.92*	78.6 *3.96*	70.6 *4.07*	72.8 *4.10*	62.5 *3.74, 3.72*
→2)-α-D-Man*p* 3 ↑ α-Abe*p*	101.1 *5.33* 101.9 *5.11*	81.0 *4.02* 64.8 *4.04*	79.0 *4.05* 34.4 *2.02, 1.98*	67.7 *4.05* 69.7 *3.88*	74.8 *3.98* 68.3 *4.12*	62.0 *3.87, 3.83* 17.0 *1.19*

and H-2/C-4 correlations. The chemical shift δ 74.8 of C-5 demonstrated the α configuration of Man*p* (compare δ 74.2 and δ 77.4 for C-5 of α-Man*p* and β-Man*p*, respectively).[29] A significant downfield displacement of the signals for Man*p* C-2 and C-3 from δ 71.5 in the non-substituted residue[29] to δ 81.0 and δ 79.0, respectively, indicated that this residue is disubstituted at positions 2 and 3.

The TOCSY spectrum showed correlations of H-1 of Gal*p* with H-2, H-3 and H-4, which were assigned by tracing connectivities in the COSY spectrum and enabled assignment of the C-2, C-3 and C-4 signals. Signals for H-5 and H-6 as well as for the corresponding carbons were assigned by a H-1/C-5 correlation in the $^{1}H,^{13}C$ HMBC spectrum, a H-5/C-5 correlation in the $^{1}H,^{13}C$ HSQC spectrum and H-5/H6a and H-5/H6b correlations in the COSY spectrum. A relatively small $J_{1,2}$ value (<3 Hz) revealed the α configuration of Gal*p*. A significant downfield displacement of the C-3 signal from δ 70.4 in the non-substituted residue to δ 78.6 demonstrated substitution of α-Gal*p* at position 3.

The $^{1}H,^{13}C$ HMBC spectrum showed the following *inter*-residue cross-peaks: Rha*p* H-1, Gal*p* C-3; Abe*p* H-1, Man*p* C-3; Gal*p* C-1, Man*p* H-2 and Man*p* H-1, Rha*p* C-4. This confirmed the substitution pattern of the monosaccharide residues and revealed their sequence in the repeating unit. All these findings were confirmed by a ROESY experiment (data not shown).

Taking the D configuration of Gal as reference, the ^{13}C NMR chemical shifts of the polysaccharide were analysed using the BIOPSEL database[31] to confirm the absolute configurations of the monosaccharides. A comparison of the observed α-glycosylation effects (δ) on the linkage carbons with the expected values showed the following relative absolute configurations of the monosaccharides in disaccharide fragments: α-Gal*p*-(1→2)-α-Man*p*—DD-pair (expected δ +9.7 for DD and δ +6.0 for DL δ +6.0, observed δ +9.5); α-Rha*p*-(1→3)-α-Gal*p*—LD-pair (expected δ +3.9 for DD and δ +8.1 for LD, observed δ +8.2); α-Man*p*-(1→4)-α-Rha*p*—DL-pair (expected δ +7.6 for DD and δ +9.2 for DL, observed δ +9.5); α-Abe*p*-(1→3)-α-Man*p*—DD-pair (expected δ +7.4 for DD and δ +5.5 for DL, observed δ +7.5). These data revealed the following absolute configurations of the monosaccharides: D for Man, L for Rha and D for 3,6-dideoxy-*xylo*-hexose (Abe).

Based on the data obtained, it was concluded that the repeating unit of the *Citrobacter* O22-polysaccharide has structure 1 as shown in Figure 2.

This structure is most closely related to that of the O-polysaccharides of *Salmonella* group B serovars.[32,33] The O-polysaccharides of *Salmonella* groups A, B and D have the same main chain with a Man→Rha→Gal trisaccharide repeat that is substituted with different 3,6-dideoxyhexoses. These unusual monosaccharides, including abequose, are found almost exclusively in O-antigenic components of bacterial lipopolysaccharides[32] and largely contribute to the serological specificity of the bacteria. So far five of eight possible isomers have been found in few bacterial species, including four (abequose, paratose, tyvelose and colitose) within the strains of *Salmonella enterica* and all five (including also ascarylose) within the strains of *Yersinia pseudotuberculosis* (for structures see Bacterial Carbohydrate Structures Database at http://www.glyco.ac.ru/bcsdb) Abequose has been found earlier in the O-antigens of *Salmonella* group B[32,33], *Salmonella* Kentucky,[34] *Citrobacter* O38[35] and *Citrobacter* strain 396[11,36] (Fig. 2). The abequose-containing O-antigen of *Citrobacter* O22 studied in this work differs from *Salmonella* group B O-antigens in the lack of glucosylation.

A number of abequose-containing LPS from *Citrobacter* and *Salmonella* were analysed by SDS–PAGE (Fig. 3A) and tested in immunoblotting with antiserum against *C. freundii* O22 cells (Fig. 3B). All LPS showed a ladder-like pattern of slow migrating high-molecular-mass LPS species with O-polysaccharide chains of different lengths as well as fast migrating bands of short-chain LPS species with no O-polysaccharide attached to the core. Anti-*C. freundii* O22 serum recognised the homologous LPS and those of *Salmonella* B strains, including serovars Typhimurium, Brandenburg, Sandiego and Paratyphi B. The other abequose-containing LPS, including those of *C. werkmanii* O38 and *Salmonella* Kentucky, did not react with this antiserum and, hence, are not serologically related. The lack of cross-reactivity is evidently accounted for by different structures of the main chain of the O-polysaccharides of these bacteria.

The LPS of *E. coli* O111 did not cross-react with anti-*C. freundii* O22 serum and, vice versa, anti-*E. coli* O111 serum recognised no abequose-containing LPS but the homologous LPS, which contains terminal α-colitose residues[37] (Fig. 3C). The lack of serological cross-reactivity is evidently due to different absolute configurations of colitose and abequose and corroborates earlier observations that 3,6-dideoxyhexoses are immunodominant sugars in bacterial O-antigens.

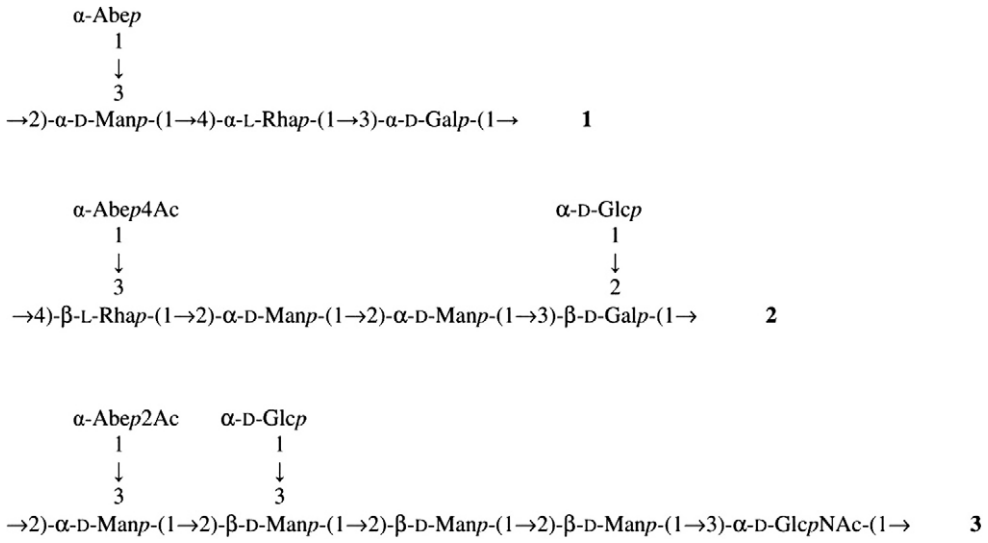

Figure 2. Structures of abequose-containing O-polysaccharides of *C. freundii* O22 (this work) and *Salmonella* group B[32,33] (**1**), *C. werkmanii* O38[35] and *Salmonella* Kentucky[34] (**2**), and *Citrobacter* strain 396[36] (**3**). In *Salmonella* group B, glucosylation is not shown. In *Salmonella* Kentucky, O-acetylation is absent and glucose is present in ~75% repeating units.

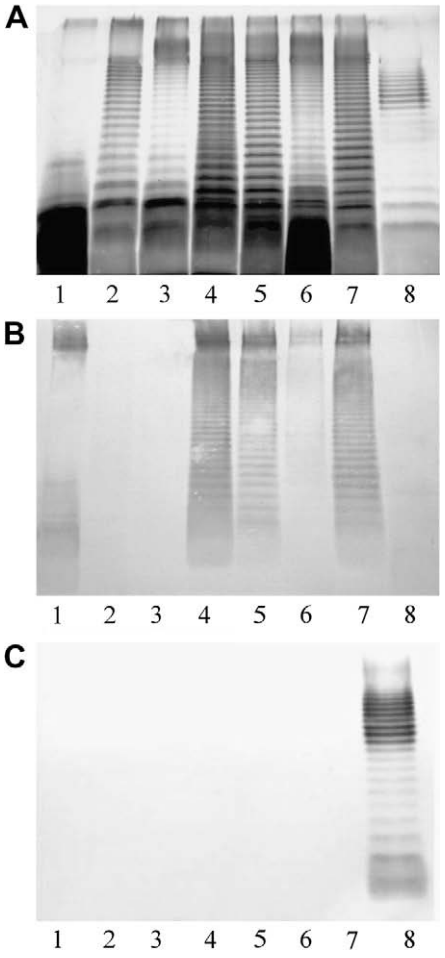

Figure 3. Silver-stained SDS–PAGE (A) and immunoblotting with anti-*C. freundii* O22 serum (B) and anti-*E. coli* O111 serum (C) of the LPS from: (1) *C. freundii* O22 (PCM 1555); (2) *C. werkmanii* O38 (PCM 1489); (3) *Salmonella* Kentucky (PCM 1720); (4) *Salmonella* Brandenburg (PCM 1734); (5) *Salmonella* Sandiego (PCM 1730); (6) *Salmonella* Paratyphi B (PCM 855); (7) *Salmonella* Typhimurium (PCM 999); (8) *E. coli* O111 (PCM 277).

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