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Note

Structure and serological analysis of the *Hafnia alvei* 481-L O-specific polysaccharide containing phosphate in the backbone chain

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Abstract—The lipopolysaccharide was extracted from cells of *Hafnia alvei* 481-L bacterial strain and, after mild acid hydrolysis, the O-specific polysaccharide was isolated and characterised. On the basis of chemical analyses and NMR spectroscopic studies of the polysaccharide and oligosaccharides obtained after Smith degradation, or hydrogen fluoride treatment, it was found that the repeating unit of the O-specific polysaccharide is a phosphorylated hexasaccharide:

$$[\rightarrow P\rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow]_n$$
$$\alpha\text{-D-Glc}p(1\rightarrow 4)\rfloor \qquad \beta\text{-D-Glc}p(1\rightarrow 3)\rfloor$$

The biological repeating unit of the *H. alvei* 481-L O-antigen has galactose phosphate at the nonreducing terminus. Serological tests indicate that this strain represents an individual serotype in the *H. alvei* genus.

Keywords: *Hafnia alvei*; Enterobacteria; O-antigen; Lipopolysaccharide; LPS; Endotoxin; Polysaccharide; Structure

Hafnia alvei microorganisms are found in soil, water and dairy products, but also have been reported as opportunistic agents in hospital infections.[1] These bacteria were shown to cause acute gastroenteritis,[2,3] diarrhoea[4,5] and respiratory tract infections.[6] This justifies studies of the lipopolysaccharide (LPS, O-antigen), an endotoxic component of cell wall of these bacteria. The LPS of Gram-negative bacteria is built up of an O-specific polysaccharide chain attached *via* a core oligosaccharide to lipid A.[7] According to the serological classification,[1,8] this species includes 39 O-serotypes. Immunochemical studies on lipopolysaccharides of this genus involved 33 strains.[9] The structures of over 20 O-specific polysaccharides have been elucidated ([10–23] and references cited therein). These polysaccharides were found to contain components unusual for bacterial LPS, such as neuraminic acid, 3-amino-3,6-dideoxy- and 4-amino-4,6-dideoxy-hexoses, 2-aminoethanol, glycerol and pentitol phosphates, ribose, uronic acids and (*R*)-3-hydroxybutyric acid.

Now we describe the structure of the O-specific polysaccharide of *H. alvei* strain 481-L, which did not

Abbreviations: LPS, lipopolysaccharide; O-PS, O-specific polysaccharide; OS, oligosaccharide; PS, polysaccharide; 1D and 2D, one- and two-dimensional; HSQC, ^{1}H-detected heteronuclear single quantum coherence spectroscopy

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express serological cross-reactivity with other serotypes of this genus.

Mild acid hydrolysis of the LPS released a lipid residue and a water-soluble carbohydrate portion, which was fractionated on Bio-Gel P-4 to give eight fractions. When mild aqueous hydrolysis of LPS was performed with 0.1 M sodium acetate buffer, pH 4.5, a polysaccharide component was obtained in high yield. The first fraction represented a high-molecular-mass O-specific polysaccharide (PS, fraction 1), fractions 4-6 contained core-related oligosaccharides and fractions 7 and 8 consisted of low-molecular-mass materials, including free 3-deoxyoctulosonic acid released from the LPS. These latter two fractions were not considered further. Fractions 2 and 3 were oligosaccharides derived from the polysaccharide as they had the same sugar composition (Table 1).

Sugar analysis by GLC–MS analysis of alditol acetates after hydrolysis with 10 M hydrochloric acid (80 °C, 30 min) of the polysaccharide material revealed the presence of glucose, galactose, GlcN and GalN in molar ratios 2.0:0.4:0.9:1.9. After solvolysis with anhydrous HF (20 °C, 3 h), the content of Gal increased suggesting phosphorylation of this monosaccharide in the polysaccharide. When the polysaccharide fraction 1 was treated with 40% HF and the product subjected to gel filtration on Bio-Gel P-4, the material was eluted as a single peak with an elution volume and sugar composition corresponding to oligosaccharide fraction 3. Phosphate and *O*-acetyl groups were identified on NMR spectra (see below). The chemical analysis data showed that the O-specific polysaccharide of *H. alvei* 481-L has a repeating unit containing two residues of Glc, two residues of GalNAc, one residue each of Gal, GlcNAc, one phosphate group (2.4% of phosphorus) and *O*-acetyl groups.

GLC–MS analysis of oligosaccharide 4 revealed the presence of Glc, Gal, GlcN, GalN and a heptose in the molar ratios 4.0:0.1:0.8:2.4:1.5, while analysis of fraction 5 revealed only two glucoses and one heptose residue. Therefore, oligosaccharide 5 is a core oligosaccharide and fraction 4 is the core substituted with one oligosaccharide O-specific repeating unit.

The results of methylation analysis of the native and dephosphorylated O-specific polysaccharide of *H. alvei* 481-L as well as of the native and dephosphorylated oligosaccharides are given in Table 2. Methylated derivatives of polysaccharide fraction 1 came from the presence of terminal glucose, 4-substituted GlcNAc, 3,4-disubstituted GalNAc residues and a small amount of 2,3,4,6-tetra-*O*-methylgalactose formed most likely by partial dephosphorylation of substituted Gal under basic conditions of the methylation procedure. Methylation analysis of the dephosphorylated O-specific polysaccharide revealed the terminal Glc and Gal residues, 4-substituted GlcNAc and 3,4-disubstituted GalNAc residues in the molar ratios 2.0:1.3:1.0:1.7, respectively. Dephosphorylation caused the appearance of the terminal galactose as a result of cleavage during HF treatment. The position of phosphorylation was determined

Table 1. Sugar analysis (in molar ratios) of the poly- and oligosaccharides released from *H. alvei* strain 481-L lipopolysaccharide

Sugar component	Fraction								
	1	2	3	4	5	6	1HF	1IO_4, HF	1Smith HF
	Molar ratio								
Glc	**2.0**	**2.0**	**2.0**	4.0	**2.0**	**2.0**	**2.0**	—	—
Gal	0.5	0.4	0.3	0.1	—	—	1.2	—	—
GlcNAc	1.0	0.8	0.8	0.8	—	0.7	0.8	**1.0**	**1.0**
GalNAc	2.2	1.6	1.8	2.4	—	2.0	1.9	1.8	2.8
Hep	—	—	—	1.5	1.0	0.5	—	—	—
Gro	—	—	—	—	—	—	—	3.0	—

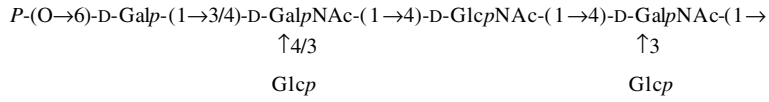
Table 2. Methylation analysis (in molar ratios) of the poly- and oligosaccharides released from *H. alvei* strain 481-L lipopolysaccharide

Sugar derivative	Fraction						
	1	2	3	4	5	2HF	1Smith
	Molar ratio						
2,3,4,6-Me_4-Glc	2.0	1.2	1.4	1.5	0.8	2.0	—
2,3,4,6-Me_4-Gal	0.4	—	—	—	—	1.3	—
2,3,6-Me_3-Glc	—	—	—	1.0	0.3	—	—
2,4,6-Me_3-Glc	—	—	—	2.5	1.0	—	—
3,4,6-Me_3-GalNAc	—	—	—	—	—	—	1.0
3,6-Me_2-GlcNAc	0.6	1.0	1.0	—	—	1.0	0.6
3,6-Me_2-GalNAc	—	—	—	—	—	—	0.3
6-Me-GalNAc	0.9	1.5	2.0	—	—	1.7	—

by dephosphorylation of the methylated polysaccharide followed by remethylation with CD_3I, and identification of 2,3,4-tri-*O*-methyl-6-*O*-[D_3]methylgalactose derivative. The position of the phosphate at *O*-6 of the galactose residue was proved, when the polysaccharide oxidised with periodate followed by dephosphorylation, revealed the presence of GlcNAc and GalNAc in a 1:2 molar ratio and the presence of glycerol but not tetritol. Methylation analysis of the oligosaccharide product of Smith degradation of the polysaccharide, revealed the presence of derivatives of terminal GalNAc, 4-substituted GlcNAc and 4-substituted GalNAc residues in molar ratios 1.0:0.6:0.3, indicating that one GalNAc residue is substituted by galactose and terminal glucose and the second GalNAc residue is substituted at *O*-3 by a second terminal glucose. In order to determine the sugar residue at the oligosaccharide reducing end, the dephosphorylated oligosaccharide fraction 3 was reduced with $NaBH_4$, hydrolysed, reduced with $NaBD_4$ and peracetylated. One of the two GalNAc residues was reduced with sodium borohydride indicating that galactosamine is at the reducing end of the repeating unit of the O-specific polysaccharide.

Methylation analysis of native fraction 5 revealed the presence of terminal and 3-substituted glucose as well as terminal heptose residues, and in dephosphorylated OS-5, additionally, 3-substituted and 3,7-disubstituted heptose residues were found. Thus the core oligosaccharide from *H. alvei* 481-L possesses the same residues as in the structure typical for most studied strains of *Hafnia*.[17,28]

Similar analysis of fraction 4 indicated that it contained the core oligosaccharide with one oligosaccharide repeating unit of the O-specific polysaccharide attached, galactose phosphate being the terminal residue. After dephosphorylation of fraction 4 resulting in cleavage of phosphate, the content of terminal galactose residue increased sharply with no significant changes in the substitution pattern of the other residues. These data allowed identification of the biological repeating unit[29] of the *H. alvei* 481-L O-specific polysaccharide as shown below. The site of attachment of the repeating unit to the core was not determined. Therefore, it was concluded that the O-PS from *H. alvei* 481-L has the structure as below, which represents also the biological repeating unit of the O-PS.



Experiments performed with MALDI-TOF mass spectrometry on the oligosaccharide fraction 3 treated with HF revealed the presence of a major quasi-molecular ion signal cationised with sodium at *m*/*z* 1135 with an associated potassium adduct peak at *m*/*z* 1151 (Δ of 16 m.u.) (Fig. 1), corroborating the chemical analysis and structural data, which confirmed the above structure. The polysaccharide fraction gave spectrum (not shown) with visible ion of only dimer of repeating units at *m*/*z* 2350.

Oligosaccharide fraction 3, dephosphorylated and reduced (3HF,BH_4), only reduced (3BH_4), and the polysaccharide fraction, all were analysed by NMR. Spectra of COSY, TOCSY, NOESY (for polysaccharide) or ROESY (for oligosaccharides), $^1H/^{13}C$ HSQC, HMBC, $^1H/^{31}P$ HMQC, $^1H/^{31}P$ HMQC–TOCSY were recorded and all signals were assigned using PRONTO program[25] (Figs. 2 and 3, and Table 3). Monosaccharides were identified on the basis of vicinal proton cou-

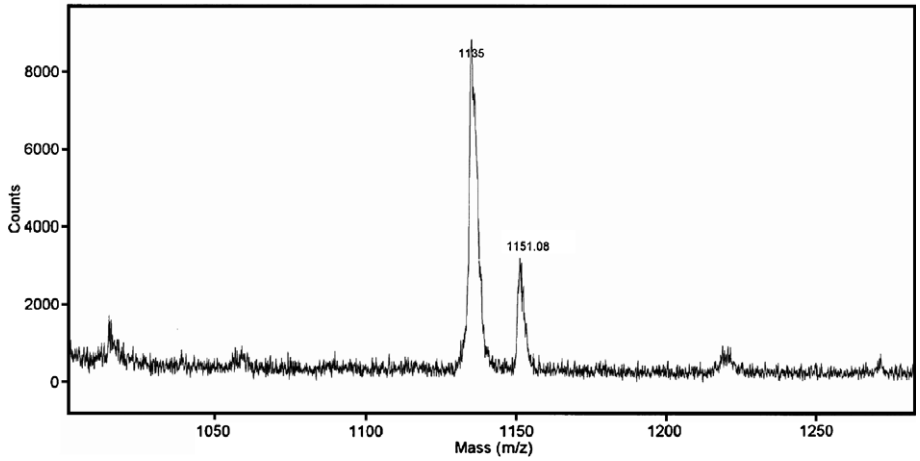


Figure 1. Part of MALDI mass spectrum of oligosaccharide fraction **3** from *H. alvei* 481-L polysaccharide treated with HF.

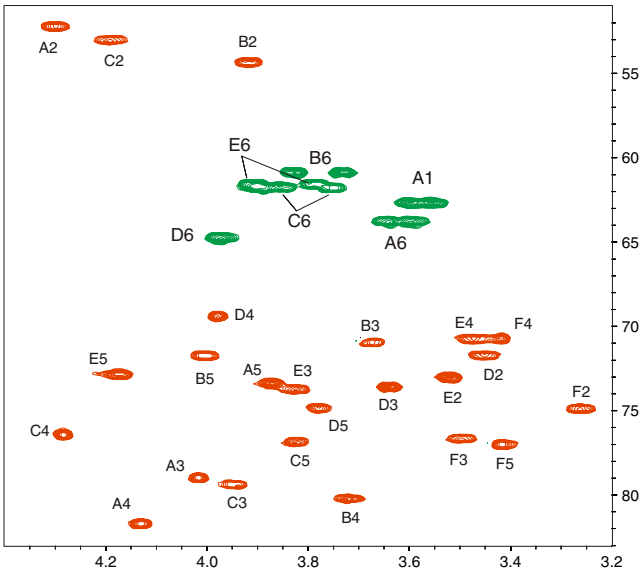


Figure 2. Fragment of HSQC spectrum of the oligosaccharide fraction $3BH_4$ from *Hafnia alvei* 481-L showing signals of the ring H/C.

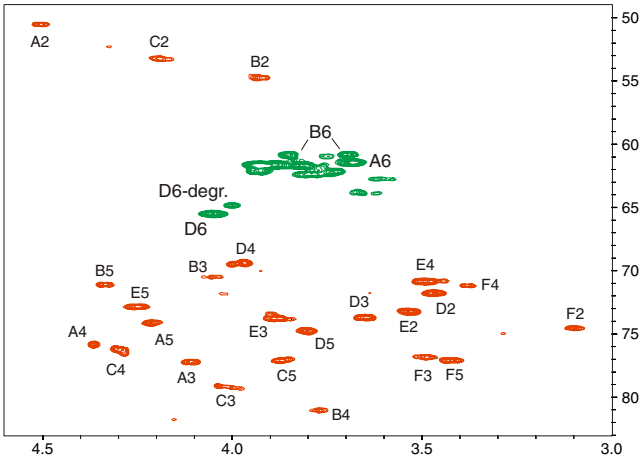


Figure 3. Fragment of the HMQC spectrum of the polysaccharide fraction from *Hafnia alvei* 481-L.

pling constants and ^{13}C NMR chemical shifts. Anomeric configurations were deduced from the $J_{1,2}$ coupling constants and chemical shifts of H-1 and C-1 signals. The β-configuration of the sugar residues D, C and F was confirmed by the observation of strong intraresidual NOE between H-1 and H-3, H-1 and H-5. Connections between monosaccharides were identified on the basis of NOE and HMBC correlations. The following NOE and H-C HMBC correlations were observed in the polysaccharide and products $3HF,BH_4$ and $3BH_4$: B1A4, E1C4, F1A3, D1C3 and C1B4, agree with the presented structures (Fig. 4). Oligosaccharide $3HF,BH_4$ had no phosphate substituents. Oligosaccharide $3BH_4$ had monophosphate at D-6 (^{31}P signal at 2.2 ppm, correlating with H-6 of galactose residue D). In the polysaccharide, repeating units were connected through phosphodiester linkages between A-1 and D-6: phosphate signal at −0.5 ppm gave HMQC correlations with H-1 of the residue A and H-6 of the residue D.

In double immunodiffusion test and SDS-PAGE with immunoblotting experiments, the rabbit serum anti *H. alvei* 481-L cells was assayed with LPS of several *H. alvei* serotypes, and the reactivity was recorded with homologous LPS (data not shown). This indicates that the strain represents individual serotype in *H. alvei* genus.

1. Experimental

1.1. Bacterial strain, preparation and degradation of LPS

Hafnia alvei strain 481-L was obtained from Dr. Z. Tyc at the National Institute of Hygiene, Warsaw. Bacteria were cultivated in liquid medium with aeration at 37 °C for 24 h, then harvested and freeze-dried. LPS was isolated by phenol-water extraction and purified on a Sepharose 2B as described previously.[9,12] The yield of LPS from *H. alvei* 481-L was 2.1% of dry bacterial mass. Other lipopolysaccharides used in this work were from previous studies.

The LPS (200 mg) was treated with 1% acetic acid (20 ml) at 100 °C for 45 min and the carbohydrate-containing supernatant was fractionated on a column (1.6 × 100 cm) of Bio-Gel P-4 into six fractions. Alternatively, LPS was degraded with 0.1 M NaOH–AcOH (pH 4.5) at 100 °C for 5 h. Gel-permeation chromatography in 0.05 M aqueous pyridine acetate buffer (pH 5.6) was monitored with a Knauer differential refractometer.

1.2. Chemical methods

Determinations of sugars, *O*-acetyl groups, phosphorus, absolute configurations of sugars using enzymatic methods, as well as dephosphorylation (48% hydrofluoric acid, 4 °C, 3 days), periodate oxidation and Smith degradation were performed as reported earlier.[10,12,15] O-Deacetylation of the O-specific polysaccharide was carried out with aqueous 12% ammonia overnight at room temperature. Solvolysis with anhydrous HF was performed at 20 °C for 3 h, as previously described.[12] For sugar analysis, hydrolysis was performed with 2 M trifluoroacetic acid at 120 °C for 2 h or with 10 M HCl at 80 °C for 30 min followed by evaporation with a stream of N_2. After the reduction with $NaBH_4$ and acetylation with Ac_2O in pyridine, the resulting alditol acetates were analysed by GLC–MS using Hewlett–Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and temperature program from 150 to 270 °C with 8 °C/min. Methylation was performed according to the Hakomori procedure[24] and

Table 3. NMR spectroscopy data for the polysaccharide and oligosaccharide fractions 3HF,BH_4 and 3BH_4 (δ, ppm)

Residue	Nucleus	1	2	3	4	5	6a/6b
GalNAc-ol	^1H	3.56/3.60	4.30	4.02	4.13	3.88	3.60/3.65
A, 3HF,BH_4; 3BH_4	^{13}C	62.9	52.4	79.2	81.9	73.6	64.0
α-GalNAc	^1H	5.52	4.50	4.10	4.36	4.21	3.68/3.68
A, PS	^{13}C	95.7	50.4	77.1	75.8	74.1	61.4
α-GlcNAc	^1H	5.08	3.92	3.68	3.72	4.00	3.73/3.83
B, 3HF,BH_4; 3BH_4	^{13}C	98.9	54.5	71.1	80.4	71.9	61.0
α-GlcNAc	^1H	5.02	3.93	4.05	3.77	4.33	3.70/3.85
B, PS	^{13}C	97.7	54.6	70.3	80.9	71.0	61.0
β-GalNAc	^1H	4.63	4.19	3.94	4.28	3.82	3.84/3.90
C, 3HF,BH_4; 3BH_4	^{13}C	102.8	53.3	79.5	76.3	77.0	61.7
β-GalNAc	^1H	4.70	4.18	4.01	4.30	3.87	
C, PS	^{13}C	103.0	53.1	79.1	76.2	77.0	
β-Gal	^1H	4.42	3.44	3.62	3.90	3.64	3.75/3.79
D, 3HF,BH_4	^{13}C	106.6	71.9	73.9	70.1	76.4	62.5
β-Gal	^1H	4.43	3.45	3.64	3.98	3.78	3.97/3.97
D, 3BH_4	^{13}C	106.6	72.0	73.9	69.7	75.1	65.0
β-Gal	^1H	4.45	3.47	3.65	3.96	3.80	4.05
D, PS	^{13}C	106.4	71.7	73.5	69.3	74.7	65.5
α-Glc	^1H	5.01	3.52	3.84	3.47	4.21	3.80/3.90
E,3HF,BH_4; 3BH_4	^{13}C	100.5	73.3	73.9	70.9	73.0	61.7
α-Glc	^1H	5.01	3.53	3.88	3.50	4.25	
E, PS	^{13}C	100.4	73.1	73.6	70.7	72.7	
β-Glc	^1H	4.53	3.26	3.50	3.42	3.42	3.76/3.89
F, 3HF,BH_4; 3BH_4	^{13}C	106.2	75.0	76.9	70.8	77.2	62.0
β-Glc F, PS	^1H	4.54	3.09	4.48	3.37	3.40	
	^{13}C	106.1	74.4	76.7	71.1	77.0	

The chemical shifts for the O-acetyl group are 2.19 ppm ^1H, 21.8 ppm ^{13}C (Me) and 175.0 ppm (CO). ^{31}P signal at −0.53 ppm in the polysaccharide, 2.2 ppm in 3BH_4.

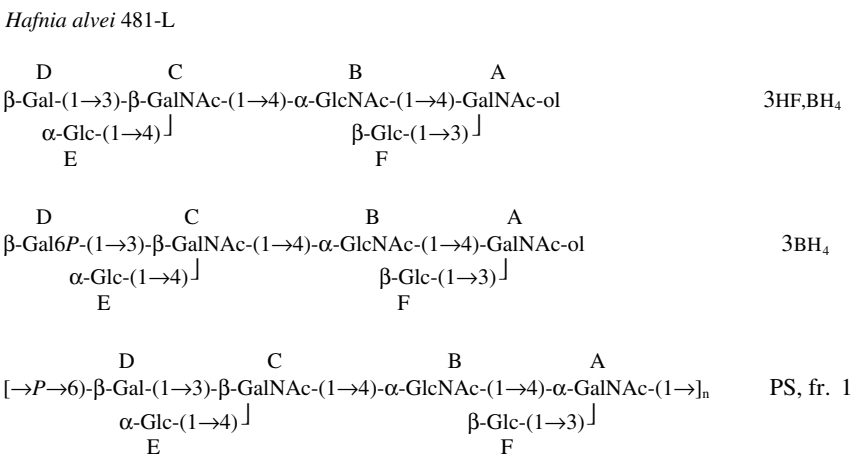


Figure 4. Structures of oligosaccharide fractions 3HF,BH_4 and 3BH_4 and the O-specific polysaccharide from *Hafnia alvei* 481-L, showing a hexasaccharide repeating unit linked with phosphate. Sugar residues are labelled according to Table 3.

methylated products were purified by extraction with 1:1 $CHCl_3$–water, then hydrolysed with 10 M HCl at 80 °C for 30 min, followed by evaporation with a stream of nitrogen, reduced with $NaBD_4$, acetylated with 1:1 Ac_2O–pyridine and analysed as above.

1.3. NMR spectroscopy

NMR spectra were recorded at 25 °C in D_2O on a Varian UNITY INOVA 500 instrument, using acetone as reference for proton (2.225 ppm) and carbon

(31.5 ppm) spectra. Varian standard programs COSY, NOESY or ROESY (mixing time of 400 ms), TOCSY (spinlock time 120 ms), HSQC and gHMBC (long-range transfer delay 100 ms) were used with digital resolution in F2 dimension <2 Hz/pt for proton–proton correlations. Spectra were assigned using PRONTO program.[25]

1.4. MALDIMS

MALDI mass spectra were recorded on a RETOF (time-of-flight) instrument from Perseptive Biosystems (Framingam, USA) equipped with a pulsed delay source extractor. Spectra were recorded from 256 laser shots (nitrogen laser, 337 nm) with an accelerating voltage of 20 kV in linear mode. 2,5-Dihydroxybenzoic acid was dissolved in aqueous 70% acetonitrile containing 0.1% CF_3CO_2H was used as a matrix; 1 μL was mixed with 1 μL of sample, placed on top of the matrix surface and allowed to dry in air. The spectra were calibrated using insulin (1 pmol/μL, *m*/*z* 5736) under the same conditions.

1.5. Serological methods

For serological tests, rabbit serum against whole cells of *H. alvei* 481-L was prepared as described previously.[26] SDS-PAGE, immunoblotting and double immunodiffusion tests were performed with LPSs as described.[11,26,27]

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