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Note

Structure of the O-polysaccharide from the LPS of a *Hafnia alvei* strain isolated from a patient with suspect yersinosis

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Abstract

The carbohydrate backbone of the *Hafnia alvei* strain Y166/91 lipopolysaccharide (LPS) was isolated by mild acid hydrolysis followed by gel permeation chromatography and studied by NMR spectroscopy and methylation analysis. Treatment with periodate and hypoiodite gave a modified polysaccharide which was also characterised. It was concluded that the polysaccharide has the following structure with two distinct regions, which are connected. The chain length parameters m and n were not determined but the ratio m/n is approximately 2.

$[\rightarrow 3)$-β-D-Gal*p*-$(1 \rightarrow 3)$-α-D-Gal*p*-$(1 \rightarrow]_m[\rightarrow 3)$-$\alpha$-D-Gal*p*-$(1 \rightarrow 3)$-$\beta$-D-Gal*f*-$(1 \rightarrow]_n$

From the present data it is not possible to determine whether it is the Gal*p*–Gal*p* chain or the Gal*p*–Gal*f* chain that is connected to the core. The structure found here is identical to that suggested for the O-specific polysaccharide chain of *Klebsiella pneumoniae* O1K2 (NCTC 5055) LPS [O. Kol, J.-M. Wieruszeski, G. Strecker, J. Montreuil, and B. Fournet, *Carbohydr. Res.*, 217 (1991) 117–125; O. Kol, J.-M. Wieruszeski, G. Strecker, B. Fournet, R. Zalisz, and P. Smets, *Carbohydr. Res.*, 236 (1992) 339–344]. © 1997 Elsevier Science Ltd.

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Diagnosis of bacterial infections are routinely made by serology, often based on bacterial carbohydrate surface antigens. In invasive infections like typhoid fever and yersinosis the titers are in general high against the lipopolysaccharide (LPS) [1]. The specificity of the serology depends on a unique carbohydrate not present in other pathogenic or commensal bacteria. If an antibody response against a bacterium is found in an ill person it is of interest to investigate the underlying reason.

Thus, a previously healthy 30 year old woman fell ill, acquired fever and other symptoms and attended ward. Initially yersinosis was suspected and faecal and serum samples were taken. In the faecal sample a bacterial strain was isolated and identified as *Hafnia alvei*. Like pathogenic *Yersinia* it was positive in

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autoagglutination at 37 °C but not at 25 °C, indicating a temperature regulated phenotype.

A preliminary ELISA was performed using the LPS from the strain as antigen and otherwise using the same conditions as in the routine *Yersinia* O3 and *Salmonella* O4 and O9 LPS-ELISA serology. It is difficult to evaluate the results of a serological test without positive and negative controls. However, the serum of the patient, taken 45 days after onset of illness, had IgG and IgM titers against the *Hafnia* LPS in the same magnitude as observed for positive cases of yersinosis and salmonellosis, in the routine serology. The IgG and IgM titers in the patient serum were both approximately 900 against the *Hafnia* LPS but < 100 against the *Yersinia* and *Salmonella* LPS. The *Yersinia* and *Salmonella* control antisera were also negative against the *Hafnia* LPS.

Some isolates of *Hafnia alvei* have been shown to be pathogenic for man, causing enteric diseases. Thus, a *Hafnia alvei* obtained from a nine months old girl with diarrhoea, was found pure in a culture. When tested in cell cultures and in rabbit ileal loops, attaching and effacing lesions (AE) were observed, similar to those associated with enteropathogenic *Escherichia coli* (EPEC) [2]. Later, using molecular biology methods, it was shown that several strains of the species *Hafnia alvei* had identical or similar virulence mechanisms as EPEC strains [3].

The present strain has not been shown to be pathogenic but the presence of antibodies directed against the LPS prompted a closer investigation and we have therefore elucidated the structure of the O-polysaccharide.

Hafnia alvei strain Y166/91 was grown on agar plates which were incubated for two days at 37 °C. The bacterial cells were harvested and isolated according to standard procedures. The Y166/91 strain was tested with polymerase chain reaction (PCR) techniques for genes that are known virulence factors of *Escherichia coli* and was negative for the heat labile toxin (LT), heat stable toxin (ST), shiga-like toxin 1 (SLT1), shiga-like toxin 2 (SLT2), for the gene corresponding to the attaching and effacing lesion (AE), and for the invasive plasmid of *Shigella*, and was not invasive in guiney pig eye (Sereny test).

The LPS was extracted from a bacterial suspension with hot phenol–water [4]. Treatment of the LPS with aq 1% acetic acid for 1 h at 100 °C released the polysaccharide (PS), which was further purified by gel-permeation chromatography, where it appeared as an excluded peak.

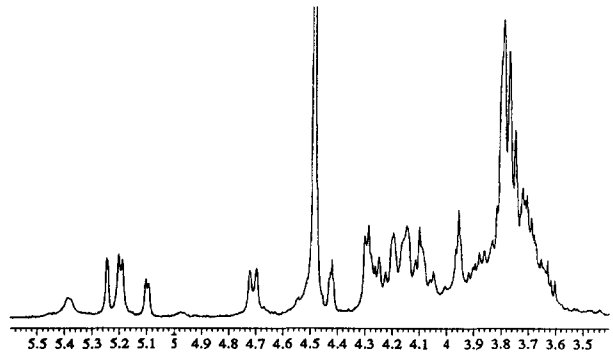

Fig. 1. The 3–6 ppm region of the ^{1}H NMR spectrum of the *Hafnia alvei* Y166/91 PS.

The ^{1}H NMR spectrum of the PS (Fig. 1) showed signals, inter alia, for four anomeric protons at δ 5.25, 5.19, 5.09, and 4.71, with the intensities of 1.0:1.9:0.9:1.8, respectively. A broader signal at δ 5.38 was also present and assigned to bacterial glycogen, which gives a resonance at that chemical shift. Treatment with amylase reduced the NMR signal at δ 5.38, further showing that the signal originated from glycogen. Some minor peaks at $\delta \sim 2$, which did not disappear after treatment with alkali, were also present.

A hydrolysate of the PS obtained using aq trifluoroacetic acid contained, apart from glucose attributed to glycogen, only galactose. GLC analysis of the acetylated (−)-2-butyl glycosides [5,6] demonstrated that galactose had the D-configuration. Methylation analysis of the PS revealed the presence of 3-substituted galactopyranose and 3-substituted galactofuranose in the molar ratio 4.4:1.0. A GLC peak attributable to chain residues in glycogen was also present. The difference in size of the signals from the anomeric protons in the ^{1}H NMR spectrum indicated an irregular structure, rather than a large repeating unit. Also, the result from the methylation analysis with a ratio of 4.4:1.0 for the derivatives is in accord with an irregularity. The two signals at δ 5.19 and 5.09 had $J_{H\text{-}1,H\text{-}2}$ values of 3.6 and 3.0 Hz, respectively, and were attributed to α-D-galactopyranose residues. The remaining two signals at δ 5.25 and 4.71 had couplings of < 1 Hz and 7.3 Hz, respectively. From the large coupling, the latter can be assigned to a β-galactopyranose residue and the signal at δ 5.25 should then correspond to the galactofuranosyl residue. Aldofuranose derivatives with β configuration have small $J_{H\text{-}1,H\text{-}2}$ values of 0–2 Hz as compared to those with the α configuration, which have $J_{H\text{-}1,H\text{-}2}$ values of 3–5 Hz [7]. The

Table 1
^{1}H and ^{13}C NMR data for the *Hafnia alvei* strain Y166/91 O-polysaccharide

Sugar residue	Chemical shifts (δ) [a]					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,6b/C-6
→ 3)-β-D-Gal*p*-(1 →	4.71 [7.3]	3.79	3.79	4.19	3.71	~ 3.78
A	(0.18)	(0.34)	(0.20)	(0.30)	(0.06)	
	104.9 [160]	70.5	78.3	65.8	75.6	61.8
	(7.5)	(−2.5)	(4.5)	(−3.9)	(−0.3)	(0.0)
→ 3)-α-D-Gal*p*-(1 →	5.09 [3.0]	3.96	3.96	4.16	4.14	~ 3.78
B	(−0.13)	(0.18)	(0.15)	(0.21)	(0.11)	
	100.2 [172]	68.0	77.8	70.0	72.0	61.8
	(7.0)	(−1.4)	(7.7)	(−0.3)	(0.7)	(−0.2)
→ 3)-α-D-Gal*p*-(1 →	5.19 [3.6]	4.09	4.15	4.29	4.26	~ 3.78
C	(−0.03)	(0.31)	(0.34)	(0.34)	(0.23)	
	96.3 [173]	68.2	79.9	69.9	71.4	61.8
	(3.1)	(−1.2)	(9.8)	(−0.4)	(0.1)	(−0.2)
→ 3)-β-D-Gal*f*-(1 →	5.25 [< 1.0]	4.42	4.11	4.28	3.89	~ 3.72
D	(−0.02)	(0.26)	(−0.01)	(0.25)	(0.03)	
	110.0 [175]	80.4	85.2	82.9	71.7	63.6
	(8.2)	(−1.8)	(8.6)	(0.1)	(0.2)	(0.0)

[a] Chemical shift differences compared to galactose are given in parentheses and $^{3}J_{H,H}$ and $^{1}J_{C,H}$ values [Hz] are given in square brackets.

galactofuranosyl residue would thus have β configuration. The ^{13}C NMR spectrum showed four signals in the anomeric region at δ 110.0, 104.8, 100.2, and 96.3, in the approximate proportions 1:2:1:2. Also this result is in agreement with an irregularity. Notably, no signals for the glycogen were observed in the ^{13}C NMR spectrum.

In order to assign the ^{1}H NMR chemical shifts $^{1}H,^{1}H$-correlated COSY and HOHAHA experiments were performed. A proton decoupled proton–carbon correlated HMQC experiment gave the ^{13}C NMR chemical shifts. The ^{1}H and ^{13}C NMR data are shown in Table 1. The sugar residues are named **A**–**D** according to increasing chemical shift of the signals from the anomeric protons. The $J_{C\text{-}1,H\text{-}1}$ values were taken from a coupled proton–carbon correlated HMQC experiment and were found to be 175 Hz (δ 110.0), 160 Hz (δ 104.8), 172 Hz (δ 100.2), and 173 Hz (δ 96.3). The anomeric configurations of the three pyranosides, are thus corroborated. For furanosides (δ 110.0) the $J_{C\text{-}1,H\text{-}1}$ values are not conclusive.

A NOESY experiment was run in order to obtain information about the sequence (Table 2). An NOE contact was observed between H-1 in the β-D-Gal*p* residue (**A**, δ 4.71) and H-3 in one of the α-D-Gal*p* residues (**C**, δ 4.15). Furthermore, the anomeric proton of residue **C** (δ 5.19) showed an NOE contact to H-3 and H-4 in residue **A** (δ 3.79 and 4.19, respectively). These NOE correlations demonstrate the presence of elements **A**–**C** and **C**–**A**. It can also be noted that the small glycosidation shifts for the signals from C-1 in **C** and C-3 in **A** and the large upfield shift of the signal for C-4 in **A** are all in agreement with a glycosidic bond in which a γ-gauche interaction is present [8]. The anomeric proton of the second α-D-Gal*p* residue (**B**, δ 5.09) had an NOE contact to H-3 in the β-D-Gal*f* residue (**D**, δ 4.11), thus, indicating the element **B**–**D**. In addition to the correlations given, a number of intra-residual correlations were obtained. No NOE correlations, however, could be

Table 2
Observed NOE correlations for *Hafnia alvei* strain Y166/91 O-polysaccharide

Anomeric proton	δ	NOE contact to proton		
Residue		δ	Intensity [a]	Residue, atom
→ 3)-β-D-Gal*p*-(1 →	4.71	3.71	m	**A**, H-5
A		3.79	s	**A**, H-3
		4.15	m	**C**, H-3
		4.19	w	**A**, H-4
→ 3)-α-D-Gal*p*-(1 →	5.09	3.96	w	**B**, H-2
B		4.11	w	**D**, H-3
→ 3)-α-D-Gal*p*-(1 →	5.19	3.79	s	**A**, H-3
C		4.09	s	**C**, H-2
		4.15	w	**C**, H-3
		4.19	s	**A**, H-4

[a] s = strong, m = medium, and w = weak intensities.

shown between the **A–C** and the **B–D** disaccharide elements. The acquired data can be interpreted in two ways, either as two disaccharide-repeating units clustered, but in the same polysaccharide, or as a mixture of two distinct polymers. In neither of the structures one would expect NOEs between the two disaccharide-repeating units. Both structures would explain the different intensities of the anomeric protons and of the anomeric carbons. Also the ratio of 4.4:1.0 for pyranose to furanose derivatives in the methylation analysis would be explained.

A long-range proton–carbon correlated HMBC experiment was run in order to confirm the results from the NOESY experiment (Table 3). A correlation from δ 4.71 (H-1 in residue **A**) to a resonance at δ 79.9 (C-3 in residue **C**) and one from δ 5.19 (H-1 in residue **C**) to a resonance at δ 78.3 (C-3 in residue **A**) confirmed the elements **A–C** and **C–A**. In addition, the signal at δ 104.9 (C-1 in residue **A**) correlated with that at δ 4.15 corresponding to the proton on the linkage carbon in **C**. A correlation from δ 5.09 (H-1 in residue **B**) to a resonance at δ 85.2 (C-3 in residue **D**) and one from δ 5.25 (H-1 in residue **D**) to a resonance at δ 77.8 (C-3 in residue **B**) confirmed the element **B–D**, and also showed, not demonstrated in the NOE experiment, the element **D–B**. In addition to the correlations given a number of intra-residual correlations were observed.

The O-specific polysaccharide from the *Klebsiella pneumoniae* O1K2 LPS (NCTC 5055) has earlier been studied. It was first suggested [9] that two separate polysaccharides were present, but subsequently it was suggested, and elegantly demonstrated, that the two disaccharide repeats were present in the same polymer [10] which was proposed to have the following structure.

$[\rightarrow 3)$-β-D-Gal$p$-$(1\rightarrow 3)$-α-D-Gal$p$-$(1\rightarrow]_m[\rightarrow 3)$-$\alpha$-D-Gal$p$-$(1\rightarrow 3)$-$\beta$-D-Gal$f$-$(1\rightarrow]_n$

The NCTC 5055 polysaccharide was submitted to a periodate oxidation [11] followed by a hypoiodite oxidation [12]. This procedure transformed the β-D-galactofuranosyl residues into β-L-arabinuronic acid residues, thus providing a substantial negative charge on the molecule. The 3-substituted α- and β-D-Gal$p$ residues would be intact. Fractionating the oxidized PS on an anion-exchange column (HPLC) showed that more than 95% of the material was retained on the column. The result would be a strong evidence for having two disaccharide-repeating units clustered in the same polysaccharide. A report [13] claiming that *Klebsiella pneumoniae* O1 LPS has two separate polysaccharides probably needs a review.

Both our ^{1}H and ^{13}C NMR data showed only minor differences to those from the *Klebsiella pneumoniae* O1K2, and we considered it possible that the two had the same structure, but that it could not be excluded that two polysaccharides were present. In order to demonstrate either of the possibilities, the polysaccharide from *Hafnia alvei* Y166/91 was subjected to the same treatment as was performed with *Klebsiella* O1K2, with periodate followed by hypoiodite. The resulting product was dialysed and passed through an anion-exchange column. Practically all the material was retained. It could not be eluted with 100 mM formic acid but was eluted with 1 M. It was evident from the NMR spectra that some of the furanosidic linkages had been hydrolysed, probably by the acidic eluent, as the signals for the **B–D** element were smaller than before. In a separate experiment non-oxidized material was run through the anion-exchange column. This material was also retained on the column but could be eluted with 100

Table 3
Observed $^{2}J_{H,C}$ and $^{3}J_{H,C}$ connectivities for *Hafnia alvei* strain Y166/91 O-polysaccharide

Anomeric atom	δ (^{1}H)	δ (^{13}C)	$J_{H,C}$ connectivities to		
Residue			δ (^{13}C)	δ (^{1}H)	Residue, atom
$\rightarrow 3)$-β-D-Gal$p$-$(1\rightarrow$	4.71		79.9		**C**, C-3
A		104.9		3.79	**A**, H-2/ H-3
				4.15	**C**, H-3
$\rightarrow 3)$-α-D-Gal$p$-$(1\rightarrow$	5.09		72.0		**B**, C-5
B			85.2		**D**, C-3
$\rightarrow 3)$-α-D-Gal$p$-$(1\rightarrow$	5.19		71.4		**C**, C-5
C			78.3		**A**, C-3
			79.9		**C**, C-3
$\rightarrow 3)$-β-D-Gal$f$-$(1\rightarrow$	5.25		77.8		**B**, C-3
D			82.9		**D**, C-4

mM formic acid. It was also noticed that the H-1 signal at δ 5.38 from the bacterial glycogen had disappeared. The resulting polymer was characterised by both ^{1}H and ^{13}C NMR, and the data are shown in Table 4. The signal for the carbonyl carbon at δ 178.4 is indicative of the Araf*A* residue. Also, downfield shifts of the H-3 and H-4 signals and the C-3 and C-4 signals in the modified residue **D** showed that Araf*A* was present. The remaining signals appeared approximately at positions previously found in the native material. The NMR data are in fair agreement with those in ref [10].

From the combined data we conclude that the PS from *Hafnia alvei* Y166/91 LPS has structure **1**. It could not be determined whether it was the **A–C** or the **B–D** region that was linked to the core-lipid A region.

A C B D

$$\left[\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow\right]_m\left[\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow\right]_n$$

1

The finding that the Y166/91 strain has the same O-polysaccharide as that of the *Klebsiella pneumoniae* type O1K2 is remarkable, but not unusual. The occurrence of the same O-polysaccharide in different species have been shown a number of times and several cases of cross-reactivity among the *Klebsiella* genus may well derive from identical O-antigenic polysaccharides. For example, *Klebsiella pneumoniae* O1 crossreacts with *Escherichia coli* O19, and *Shigella dysenteriae* 3 with *Escherichia coli* O124 [14]. Cross-reactivity within Enterobacteriaceae is common and it is likely that also other strains within Enterobacteriaceae crossreacts with the *Hafnia* strain.

The disaccharide element $\rightarrow$ 3)-α-D-Gal*p*-(1 $\rightarrow$ 3)-β-D-Gal*f*-(1 $\rightarrow$ constitutes the repeating unit in other lipopolysaccharides, e.g. in those from *Pasturella hemolytica* serotypes T4 [15] and T10 [16] and from *Serratia marcescens* O20 [17]. No data on the presence of a polysaccharide with $\rightarrow$ 3)-β-D-Gal*p*-(1 $\rightarrow$ 3)-α-D-Gal*p*-(1 $\rightarrow$ repeating units were reported in these studies.

The growth of the *Hafnia alvei* strain in the faecal sample of the patient makes it reasonable to assume that the antibody response was due to an infection with this bacterium in spite of the observation that the *Hafnia* strain has an O-antigen identical to *Klebsiella* O1. Investigations to determine the frequency of antibody response and presence in faecal samples are in progress.

1. Experimental

General methods.—Concentrations were performed by flushing with air at 50 °C. For GLC, a

Table 4
^{1}H and ^{13}C NMR data for periodate and hypoiodite oxidised *Hafnia alvei* strain Y166/91 at 18 °C

Sugar residue	Chemical shifts (δ) [a]					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,6b/C-6
$\rightarrow$ 3)-β-D-Gal*p*-(1 $\rightarrow$	4.70 [7.3]	3.81	3.79	4.21	3.72	3.77
A	(0.17)	(0.36)	(0.20)	(0.32)	(0.07)	
	105.2	70.7	78.5	66.0	75.8	61.7 [b]
	(7.8)	(−2.3)	(4.7)	(−3.7)	(−0.1)	(−0.1)
$\rightarrow$ 3)-α-D-Gal*p*-(1 $\rightarrow$	5.25 [$\approx$ 2.5]	3.98	4.02			
B	(0.03)	(0.20)	(0.21)			
	99.9	68.3	78.5			
	(6.7)	(−1.1)	(8.4)			
$\rightarrow$ 3)-α-D-Gal*p*-(1 $\rightarrow$	5.20 [3.6]	4.08	4.17	4.31	4.27	3.77
C	(−0.02)	(0.30)	(0.36)	(0.36)	(0.24)	
	96.6	68.3	80.1	70.1	71.6	61.7 [b]
	(3.4)	(−1.1)	(10.0)	(−0.2)	(0.3)	(−0.3)
$\rightarrow$ 3)-β-L-Araf*A*-(1 $\rightarrow$	5.33 [n.r.]	4.46	4.20	4.56		
D	110.9	80.1	87.5	83.8	178.4	

[a] Chemical shift differences compared to galactose are given in parentheses and $^{3}J_{H,H}$ values [Hz] are given in square brackets. n.r. not resolved.
[b] A smaller peak at δ 62.2 is also present.

Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates were performed on a DB-5 capillary column, using the temperature programme 160 °C (1 min) → 250 °C at 3 °C/min. GLC–MS (EI) was performed on a Delsi Di200 GC-Nermag R10-10H quadrupole mass spectrometer. Partially methylated alditol acetates were separated on a DB-5 or a SP-2330 capillary column, using the temperature programmes 130 °C (1 min) → 250 °C at 3 °C/min or 140 °C (1 min) → 250 °C at 3 °C/min, respectively. The absolute configuration of Gal was determined using the acetylated (−)-2-butyl glycosides according to the procedures of Leontein et al. [6] and of Gerwig et al. [5]. Gel-permeation chromatography was performed on Bio-Gel P-4 columns (Bio-Rad, USA) using water as eluent. Fractions were monitored by a differential refractometer.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70 °C (^{13}C) and 55 °C (^1H), unless otherwise stated, with a JEOL EX270 instrument, except for the HMBC spectrum which was run on a Varian Unity 500 instrument. Chemical shifts are reported in ppm, using acetone (δ_C 31.00) and sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) as internal references. $^1H,^1H$-COSY and NOESY experiments were performed using JEOL standard pulse-sequences. $^1H,^1H$-HOHAHA experiments were performed in the phase-sensitive mode. The mixing time in the NOESY experiment was 100 ms and $^1H,^1H$-HOHAHA experiments were obtained using mixing times of 40, 80, 120, and 180 ms. Proton–carbon correlated spectra (HMQC) were obtained with or without decoupling and the long-range proton–carbon correlated spectra (HMBC) were performed using a delay time of 62.5 ms.

Bacteria.—*H. alvei* strain Y166/91 was grown on agar plates containing tryptic soy broth (Difco) supplemented with 1.5% bact-agar (Difco). The agar plates were incubated for two days at 37 °C. The bacterial cells were harvested, killed with formaline and washed three times in phosphate buffered saline.

Preparation of LPS.—Bacterial cells suspended in water (300 mL) were extracted with aq 90% phenol (300 mL) under vigorous stirring at 72 °C for 1 h. After cooling, dialysis, and centrifugation, the supernatant solution was freezedried. RNA was removed by treatment with RNAse A. The LPS (500 mg) was suspended in 0.1 M NaOAc buffer (25 mL, pH 5.4). To the solution aq 0.1 M $CaCl_2$ (250 μL) and RNAse A (10 mg) were added. The pH was kept below 6 and 2 drops of $CHCl_3$ were added. The solution was kept for 3 h at 37 °C without stirring. The LPS was recovered after dialysis.

Isolation of PS.—A suspension of the LPS (50 mg) in water (5 mL) was placed in an ultrasonic bath for 5 min. Then, glacial HOAc was added to a final concentration of 1% and the solution was kept for 1 h at 100 °C. After cooling, a precipitate was obtained which was removed by centrifugation. The pellet was resuspended once in water and the solution was centrifuged. The two supernatants were combined and lyophilised to yield the PS. The PS was further purified by gel-permeation chromatography on a column of Bio-Gel P-4 and eluted with the void volume.

Sugar and methylation analysis.—Hydrolysis of native and methylated LPS and PS was performed by treatment with 2 M CF_3CO_2H at 120 °C for 2 h or with 0.5 M CF_3CO_2H at 80 °C for 16 h. The methylated products were purified on Sep-Pak C_{18}-cartridges using CH_3CN as eluent. The sugars in hydrolysates were converted into alditol acetates and partially methylated alditol acetates according to standard methods.

Periodate oxidation.—*H. alvei* PS (20 mg) was dissolved in 0.1 M NaOAc buffer, pH 3.9 (20 mL). Thereafter, $NaIO_4$ was added to a final concentration of 0.03 M and the sample was left in the dark for 72 h at 4 °C. Then, excess $NaIO_4$ was destroyed with ethylene glycol (200 μL) and the sample was recovered after dialysis.

Hypoiodite oxidation.—The periodate-oxidized material (18 mg) was dissolved in H_2O (6 mL). A small part of the material (~ 1 mg) was insoluble and was removed by centrifugation. An iodine solution (2 mL, 0.1 M; made by mixing of 0.25 M KI and 0.05 M I_2) and 0.1 M NaOH (3 mL) were then added dropwise. This was repeated until 11 mL of the iodine solution and 16.5 mL of the NaOH solution had been added. Shortly after the last addition, the mixture was neutralised with 0.2 M HCl. The product was recovered after dialysis and run through a DEAE-Sepharose anion-exchange column (1 × 15 cm) which was eluted first with 100 mM HCO_2H and then with 1 M HCO_2H.

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