Structure of an Atypical O-Antigen Polysaccharide of *Helicobacter pylori* Containing a Novel Monosaccharide 3-*C*-Methyl-D-mannose†

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ABSTRACT: Lipopolysaccharides (LPS) were isolated by hot phenol−water extraction from Danish *Helicobacter pylori* strains D1, D3, and D6, which were nontypeable using a variety of anti-Lewis and anti-blood-group monoclonal antibodies. An atypical O-chain polysaccharide (PS) was liberated from the LPS of the three strains by acid under mild conditions and found to contain D-rhamnose (D-Rha), L-rhamnose (L-Rha), and a branched sugar, 3-*C*-methyl-D-mannose (D-Man3*C*Me). The last sugar, which has not hitherto been found in Nature, was identified using GLC-MS of the derived alditol acetate and the partially methylated alditol acetate, and ^{1}H and ^{13}C NMR spectroscopy, including NOESY and ^{1}H,^{13}C HMBC experiments. The following structure of the trisaccharide repeating unit of the PS was established: →2)-α-D-Man*p*3*C*Me-(1→3)-α-L-Rha*p*-(1→3)-α-D-Rha*p*-(1→. In contrast to the pathogenic importance of the Lewis antigen mimicry exhibited by the PS of *H. pylori* strains previously investigated, the biological relevance of the atypical PS for *H. pylori* pathogenesis is unclear. The production of a differing surface PS may represent a form of antigenic variation by these particular *H. pylori* strains and/or may reflect the adaptation of these strains to a particular human population.

Helicobacter pylori is a prevalent pathogen of humans (*1*), and infection is associated with a broad spectrum of clinical outcomes including the development of gastritis, gastric and duodenal ulcers, and gastric cancer (*2*). Like the outer membrane of other Gram-negative bacteria, that of *H. pylori* contains lipopolysaccharides (LPS).[1] As these molecules comprise an important group of bacterial cell surface glycolipids which interact with the bacterial microenvironment and the infected host, attention is focusing on the structure of *H. pylori* LPS and its contribution to pathogenesis (*3*). The structure of the lipid A moiety of *H. pylori* LPS has been established (*4*), and the low phosphorylation and unusual acylation pattern in this LPS region contribute to the low endotoxic activity and low immunological response observed with *H. pylori* LPS (*3, 5*).

Structural studies on LPS of certain *H. pylori* strains have shown that the O-chain polysaccharides (PS) exhibit mimicry of Lewis^{x} (Le^{x}) and/or Lewis^{y} (Le^{y}) blood group antigenic determinants (*6*−*12*). These are formed by mono- or difucosylated *N*-acetyl-*β*-lactosamine units attached to the LPS core, or at the nonreducing end of the PS. Typically, these PS have a poly(*N*-acetyl-*β*-lactosamine) chain decorated with multiple lateral α-L-fucose residues (*8*−*12*) or, in some strains, with additional glucose or galactose residues (*7, 13*). Furthermore, Lewis^{a} (Le^{a}), Lewis^{b} (Le^{b}), and H type I antigenic determinants in the PS of other *H. pylori* strains have been found (*6*).

Although Le^{x}, Le^{y}, and related blood group antigens are present in the human gastric mucosa (*14*), the pathogenic relevance of Lewis antigen mimicry by *H. pylori* remains unclear. Upon initial infection, it has been suggested that expression of Lewis antigens may camouflage the bacterium or aid bacterial adhesion (*15, 16*). In prolonged chronic infection by *H. pylori*, expression of Le^{x} and Le^{y} antigens has been implicated in the pathogenesis of atrophic gastritis by the induction of autoreactive antibodies (*15, 17*), but this pathogenic mechanism has not been unequivocally established (*18, 19*). Also, evidence suggests that expression of Lewis antigens by *H. pylori* may influence the inflammatory response to infection (*16, 17, 20*).

Serotyping *H. pylori* strains based on a panel of anti-Lewis monoclonal antibodies has been proposed (*21*), and various surveys using such antibodies have indicated that up to 85% of strains express Le^{x} or Le^{y} antigens (*20*−*22*). Despite the description of other Lewis antigen-related structures in the PS of a limited number of *H. pylori* strains (*6, 7, 13*),

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[1] Abbreviations: EI, electron impact; HMBC, heteronuclear multiple-bond connectivity; GPC, gel permeation chromatography; Le^{x}, Lewis X (similarly for other Lewis antigens); LPS, lipopolysaccharide; Man, mannose; Man3*C*Me, 3-*C*-methylmannose; PS, O-chain polysaccharide; Rha, rhamnose.

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information has been lacking on the PS of the remaining strains that are nontypeable with antibodies toward Lewis and other blood group determinants. In this paper, we report the structure of a different PS type which contains no typical *H. pylori* PS components, but D-rhamnose and L-rhamnose, and a branched sugar, 3-*C*-methyl-D-mannose, which hitherto has not been found in Nature.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Cultivation, Isolation, and Degradation of Lipopolysaccharide. H. pylori strains D1, D3, and D6 were isolated from gastroduodenal biopsies of patients at the Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark. Serological analysis by both Western blotting and an enzyme-linked immunosorbent assay with monoclonal antibodies against a variety of Lewis antigens (anti-Le^x, -Le^y, -sialyl-Le^x, -Le^a, -Le^b, or -H type I) or against blood group determinants (anti-A, -B, or -AB), as described previously (*12*), showed that the strains were nontypeable. The bacterial strains were grown on blood agar to produce biomass as described previously (*23*). Preparations of LPS were isolated by extraction of biomass with hot aqueous phenol (*24*) in yields of 3.5−4.1% (dry weight), and degraded with 0.1 M sodium acetate buffer, pH 4.2, for 2 h at 100 °C. The water-soluble carbohydrate portion was fractionated by gel-permeation chromatography (GPC) on a column (70 × 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate, pH 4.5, as eluent and a flow rate of 0.5 mL/min. Monitoring was performed with a Waters differential refractometer, and 10 mL fractions were collected.

Sugar Analysis. Hydrolysis was performed with 2 M trifluoroacetic acid (120 °C, 2 h, or 100 °C, 1 h, for quantitative hydrolysis of the 3-*C*-methyl-D-mannose residue). Monosaccharides were identified by GLC as alditol acetate derivatives (*25*) using a Hewlett-Packard 5880 instrument on a DB-5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. The absolute configurations of the rhamnose residues were determined by GLC of the acetylated (+)-2-octyl glycosides (*26*).

Methylation Analysis. Methylation was performed using methyl iodide in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide (*27*). Hydrolysis was performed as for sugar analysis, and subsequently partially methylated monosaccharides were reduced with $NaBH_4$, converted to alditol acetates, and analyzed by GLC-MS on a Hewlett-Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer, using the above DB-5 column and a temperature gradient of 130 °C (1 min) to 250 °C at 3 °C/min. Identification of the Rha derivatives was carried out using published data (*28*), and of the 3-*C*-methyl-D-mannose derivative by applying general rules for fragmentation of partially methylated alditol acetates (*29*).

Smith Degradation. The PS was oxidized with 0.1 M $NaIO_4$ (0.5 mL) at 20 °C for 48 h in the dark; an excess of the oxidant was destroyed by addition of ethylene glycol (0.03 mL). The product was reduced with an excess of $NaBH_4$ (20 mg) at 20 °C for 16 h, desalted by GPC on Sephadex G-50, and hydrolyzed with 2% acetic acid (2 h, 100 °C), and the resulting oligosaccharide was isolated by chromatography on Sephadex G-50. The oxidation and reduction were repeated once with this oligosaccharide to yield a glycoside of D-Rha.

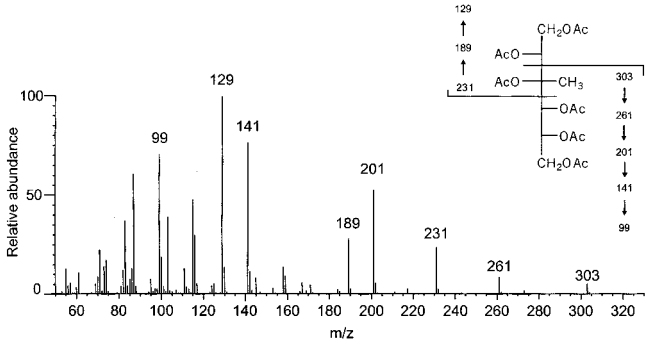

FIGURE 1: Significant fragments observed for 3-*C*-methylmannitol hexaacetate.

NMR Spectroscopy. NMR spectra of the PS from *H. pylori* D6 (9 mg) in 600 μL of 99.96% D_2O were recorded at 50 °C using a Varian Inova 600 MHz NMR instrument. Chemical shifts are reported in ppm relative to internal sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propanoate (δ_H 0.00) or external dioxane (δ_C 67.40) as references. Data processing was performed using Varian VNMR software. Double-quantum-filtered $^1H,^1H$-correlated spectroscopy (DQF-COSY) (*30*), heteronuclear single-quantum coherence (HSQC) (*31*), heteronuclear multiple-bond connectivity (HMBC) (*32*), and NOESY (*33*) experiments were used to assign signals and performed using standard pulse sequences. A mixing time of 100 ms was used in a NOESY experiment, and a 50 ms delay for the evolution of long-range couplings in a gHMBC experiment.

RESULTS AND DISCUSSION

Mild acid degradation of the LPS from *H. pylori* strains Danish 1, 3, and 6 resulted mainly in a polysaccharide (PS) fraction which was separated from a smaller amount of an unsubstituted core oligosaccharide by GPC. The PS yields were 28%, 17%, and 29% calculated from the dry weight of LPS, from strains D1, D3, and D6, respectively. Sugar and methylation analyses and NMR spectroscopic studies showed that the PS from the three strains had the same composition and structure, and only results from strain D6 are stated.

After complete acid hydrolysis of the PS, sugar analysis of the liberated monosaccharides using GLC of the derived alditol acetates revealed mainly Rha. Determination of the absolute configuration of the Rha residues, by GLC analysis of the (+)-2-octyl glycosides (*26*), demonstrated that both the D- and L-isomers were present in equal amounts. In addition, GLC analysis showed the presence of smaller amounts of Man and an unknown monosaccharide [identified as 3-*C*-methyl-D-mannose (D-Man3*C*Me), see below], as well as trace amounts of Glc and Gal. The unknown sugar was partially destroyed during strong hydrolysis with 2 M trifluoroacetic acid, but survived to a larger extent under milder hydrolytic conditions; the Rha:Man:Man3*C*Me ratios were 1:0.34:0.11 when strong hydrolysis conditions were used (120 °C, 2 h), and 1:0.21:0.29 when milder conditions were employed (100 °C, 1 h).

The EI mass spectrum (Figure 1) of the third sugar clearly showed that it was an acetylated 3-*C*-methylhexitol, which could be derived from either a 3-*C*-methylhexose or a 4-*C*-

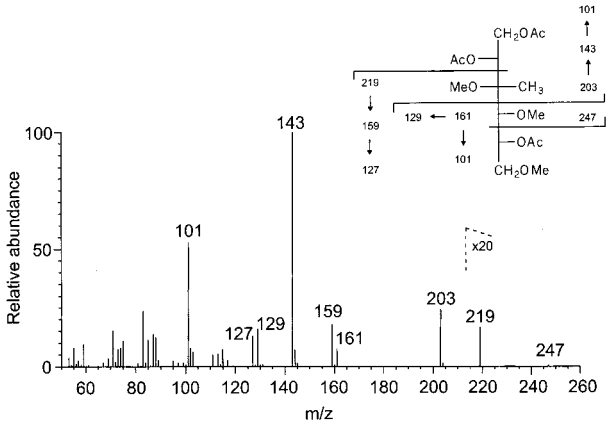

FIGURE 2: Significant fragments observed for 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-3-*C*-methylmannitol.

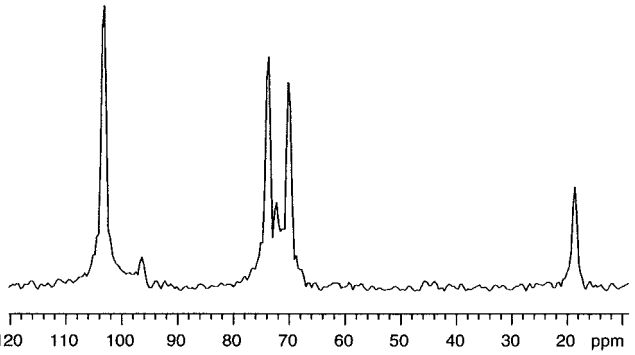

FIGURE 3: F_1-slice at δ_H 3.76 in the $^1H,^{13}C$ HMBC spectrum of the PS from *H. pylori* D6 showing ^{13}C signals resulting from correlations from H2 in 3-*C*-methylmannose (residue **A**).

methylhexose. As fragmentation is preferred next to a methylated or methoxylated position (*29*), the primary fragment ions at *m*/*z* 231 and 303 are characteristic, and correspond to the C1−C3 and C3−C6 fragments, respectively. The secondary ions at *m*/*z* 261, 201, 189, 141, 129, and 99 were derived from these two by losses of CH_2═CO and AcOH.

Methylation analysis revealed 3-substituted Rha as the main component of the PS, with smaller amounts of terminal and 2-substituted Man, and 2-substituted Man3*C*Me (the location of the *C*-methyl group was determined from NMR, see below). In the GLC-MS analysis of the methylated derivatives, the Man3*C*Me derivative gave a spectrum that is typical of partially methylated alditol acetates with more abundant methyl-containing ions resulting from the cleavage of C−C linkages at the methylated carbon, i.e., C3 (Figure 2). Ions were observed, inter alia, for fragments C1−C3, C3−C6, C4−C6, and C1−C4 at *m*/*z* 203, 219, 161, and 247, respectively. Therefore, *O*-methyl groups were located at positions 3, 4, and 6, and an *O*-acetyl group was located at position 2, thus demonstrating that the Man3*C*Me residue is 2-substituted. Methylation analysis also showed that all sugar residues were in the pyranoid form.

The ^1H NMR spectrum showed, inter alia, signals for three anomeric protons at δ 5.14, 5.04, and 5.03, all with small $J_{1,2}$ values, and for methyl groups at δ 1.42 (s), 1.32 (d), and 1.30 (d), suggesting the PS to contain three sugars in the repeating unit. This was confirmed from the ^{13}C NMR spectrum which showed 19 signals in agreement with 2 Rha residues and 1 Man3*C*Me residue, as deduced from chemical analysis data. The ^1H- and ^{13}C NMR signals were assigned using two-dimensional homo- and heteronuclear correlated experiments, and the results are shown in Table 1. The residues are denoted **A**, **B**, and **C** according to decreasing ^1H NMR chemical shifts of the signals from the anomeric protons. Residue **A** was identified as Man3*C*Me, and **B** and **C** were Rha residues, as shown by their spin systems. All residues showed nonresolved $J_{H1,H2}$ couplings and $J_{H1,C1}$ values of ∼171 Hz. Thus, all sugar residues in the PS are α-linked pyranosides.

The ^1H and ^{13}C NMR chemical shifts of residue **A** were in agreement with a 3-*C*-methylhexose. The stereochemistry of **A** was determined by a combination of $^1H,^{13}C$ HMBC (Figure 3) and $^1H,^1H$ NOESY (Figure 4) experiments. The small $J_{H1,H2}$ value indicates an axial hydroxyl group at C2. A large $J_{H4,H5}$ value indicated a trans-diaxial relationship between H4 and H5. This reduces the alternatives to the *manno* and the *altro* configurations. The configuration at C3 was determined by NOE spectroscopy. From the methyl group (δ_H 1.42), NOE correlations were observed to H2 and H5 in the same residue, but not to H4. This demonstrated that the methyl group is axial, and consequently that the hydroxyl group at C3 is equatorial. Therefore, residue **A** is a 3-*C*-methylmannopyranosyl residue. In the HMBC spectrum (Figure 3), correlations were observed in residue **A**, inter alia, from the protons of the methyl group to C2, C3, and C4; from the carbon of the methyl group to H2 and H4; from C6 to H5; and from H2 to C1, C3, and C4. These data corroborate the attachment of the methyl group to C3.

Table 1: Chemical Shifts (δ) of the Signals in the ^1H and ^{13}C NMR Spectra of the O-Chain Polysaccharide from *H. pylori* D6

	$^1H/^{13}C$						
sugar residue	1	2	3	4	5	6	3*C*Me
→2)-α-D-Man*p*3*C*Me-(1→ (**A**)	5.14 [171][a]	3.76	−	3.79 {10.4}[c]	3.89	3.78,3.88	1.42
	96.3	83.8	73.9	70.2	72.5	62.1	19.0
→3)-α-L-Rha*p*-(1→ (**B**)	5.04 [171]	4.19	3.96	3.55	4.00	1.30	
	(−0.08)[b]	(0.27)	(0.15)	(0.10)	(0.14)	(0.02)	
	96.9	67.3	74.9	71.1[d]	69.7	17.5[e]	
	(2.1)	(−4.5)	(3.9)	(−2.1)	(0.6)	(−0.2)	
→3)-α-D-Rha*p*-(1→ (**C**)	5.03 [172]	4.31	3.87	3.56	3.83	1.32	
	(−0.09)	(0.39)	(0.06)	(0.11)	(−0.03)	(0.04)	
	103.2	66.9	75.2	71.0[d]	70.1	17.7[e]	
	(8.4)	(−4.9)	(4.2)	(−2.2)	(1.0)	(0.0)	

[a] $J_{C1,H1}$ values (Hz) are given in brackets. [b] Chemical shift differences to α-Rha*p* are given in parentheses. [c] $J_{H4,H5}$ value (Hz). [d,e] Pairwise interchangeable.

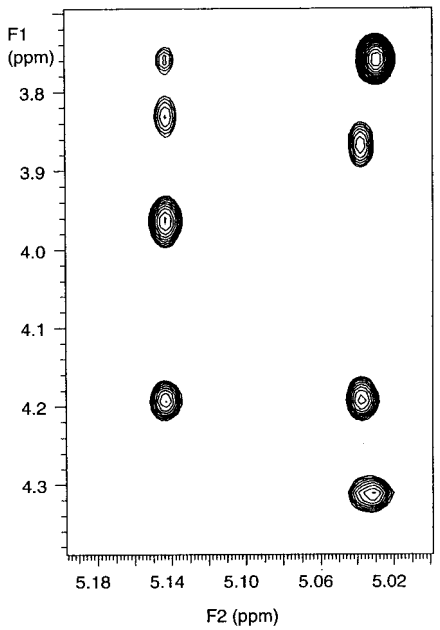

FIGURE 4: Part of a $^1H,^1H$ NOESY spectrum of the O-chain polysaccharide from *H. pylori* D6 showing correlations from anomeric protons (F_2-dimension).

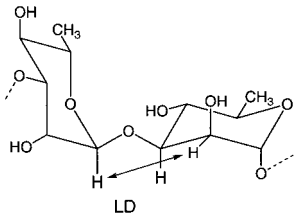

FIGURE 5: LD sequence of Rha elements **B** and **C** where the γ-gauche interaction is present as indicated by an arrow.

The sequence of sugars was determined, also by NOE and HMBC, as -**A**-**B**-**C**-. The HMBC spectrum (Figure 3) showed, inter alia, correlations between the following atoms: C1 in residue **A** and H3 in residue **B**, C1 in residue **B** and H3 in residue **C**, C1 in residue **C** and H2 in residue **A**, demonstrating all three glycosidic linkages. Further corroboration was obtained from the correlation between H1 in residue **C** and C2 in residue **A**. The NOESY spectrum (Figure 4) showed the expected cross-peaks and also confirmed the sequence of sugar residues. Therefore, the PS of the *H. pylori* strains D1, D3, and D6 is linear and has a trisaccharide repeating unit.

The absolute configurations of the sugar residues in the PS were analyzed first by NMR spectroscopy, whereby the relative configurations of all sugar residues were deduced, and second by a double Smith degradation, in which a 4-deoxytetritol rhamnoside was obtained, for which the absolute configuration of Rha could be determined. Together with information on the relative configurations, all absolute configurations could be established.

A relatively small glycosylation shift of the signal from C1 in residue **B** and a significant upfield shift of the signal from C2 in **C** (Table 1) showed that a γ-gauche interaction occurs in these residues (*34*) (Figure 5). This is in agreement with different absolute configurations, i.e., LD or the reverse, DL. A glycosidic linkage with a γ-gauche interaction is also present for sugars **A** and **B** (Figure 6), and the absolute configurations must therefore also be either DL or LD.

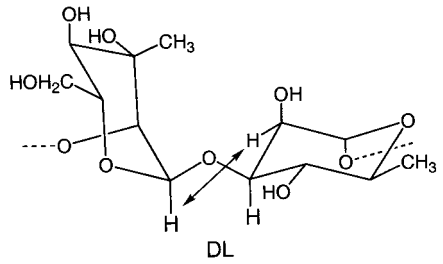

FIGURE 6: Disaccharide element **A**-**B** showing the γ-gauche interaction with an arrow.

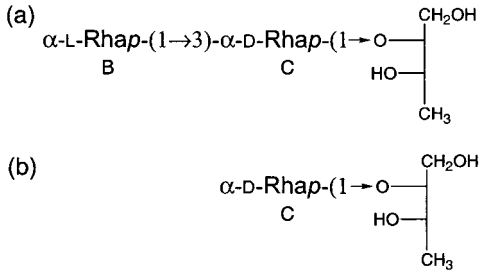

FIGURE 7: Oligosaccharides obtained from the first (a) and second (b) Smith degradation.

Besides the transglycosidic NOE between H1 in residue **C** and H2 in residue **A**, an additional NOE was observed between H5 in residue **C** and H1 in residue **A**, which can be anticipated when the two residues have the same absolute configuration (*35*). For the **A**-**B**-**C** trisaccharide element, the absolute configurations is thus either DLD or LDL.

To determine the absolute configurations of the sugars, only one residue needs to be determined as the configurations of the others will automatically follow, as discussed above. A repeated Smith degradation (Figure 7), involving treatment with periodate, borohydride, and acid under mild conditions, was chosen which would leave only residue **C** intact after completion. The first step in the Smith degradation resulted in oxidation and cleavage of Man3*C*Me (residue **A**). The Rha disaccharide−4-deoxytetritol glycoside thus obtained was repeatedly oxidized by periodate to cleave residue **B** to eventually give a 4-deoxytetritol rhamnoside.

GLC analysis of the acetylated (+)-2-octyl glycosides derived from the remaining residue **C** showed that it had the D configuration, and that consequently the sugars in the sequence **A**-**B**-**C** have the absolute configurations DLD, respectively. Therefore, the PS of *H. pylori* strains D1, D3, and D6 has a trisaccharide repeating unit with the following structure:

→2)-α-D-Man*p*3*C*Me-(1→3)-α-L-Rha*p*-(1→3)-α-D-Rha*p*-(1→
A **B** **C**

Compared with the LPS of other *H. pylori* strains previously studied, this trisaccharide repeating unit is an unusual feature of the Danish strains. The majority of *H. pylori* strains examined previously by structural and serological analyses exhibit mimicry of Le^x and/or Le^y (*8*−*12*, *20*−*22*), related structures substituted with glucose or galactose residues (*7*, *13*), or mimicry of Le^a, Le^b, and H type I antigenic determinants (*6*) in their PS component of LPS. Also, serological analysis has indicated the expression of the blood group A determinant in some *H. pylori* strains (*36*), but this

awaits confirmation in structural studies. The Danish strains examined in this study conformed to all the usual morphological and biochemical characteristics of *H. pylori* as presently established (*37*), and, moreover, 16S rRNA analysis and DNA−DNA hybridization studies confirmed identity with *H. pylori* (Moran, unpublished). The patients from which the strains were isolated suffered from gastritis, duodenitis, and esophagitis, conditions associated with *H. pylori* infection.

Lewis antigen mimicry by *H. pylori* has been implicated in camouflage of the bacterium in the gastric mucosa, bacterial adhesion, induction of autoimmunity in the development of gastritis, and influencing the inflammatory response associated with *H. pylori* infection (*15*−*17*, *20*). The biological relevance of the PS type described in this study for *H. pylori* pathogenesis is unclear. It can be speculated that production of a differing surface PS may represent a form of antigenic variation by these particular *H. pylori* strains compared with other strains, and/or reflect the adaptation of these strains to a particular human population as has been reported for other pathogenic factors (*38*).

To our knowledge, 3-*C*-methyl-D-mannose has hitherto not been found in Nature. The number of branched sugar components of lipopolysaccharides is limited, the first observed as late as in 1983 (*39*). In *Coxiella burnetii*, 6-deoxy-3-*C*-methyl-D-gulose and 3-*C*-hydroxymethyl-L-lyxose have been found (*40*), and in *Yersinia*, two 3,6-dideoxy-4-*C*-(1-hydroxyethyl)-D-*xylo*-hexoses, yersiniose A and yersiniose B (*39*). *Pseudomonas caryophylli* produces two branched C_9-sugars, one of them with a carbocyclic ring (*41*). Two not fully characterized sugars have also been found in *Nitrobacter hamburgiensis* (*42*) and *Shewanella* (Widmalm, unpublished). Another unusual feature of the PS is the simultaneous occurrence of both L-Rha and D-Rha, that has been previously reported only for two O-chain polysaccharides of a plant pathogenic bacterium, *Pseudomonas syringae* (*43*, *44*).

Signals for the core constituents and Man residues were observed in the ^{1}H NMR spectrum of the PS, but they were minor and their linkages could not be traced in the two-dimensional spectra. Therefore, the structure of the core region and the location of the mannose residues, whether they are core components or not, remain undetermined and are the subject of future investigations.

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