Carbohydrate Research 345 (2010) 830–838

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Identification of cell-surface mannans in a virulent *Helicobacter pylori* strain

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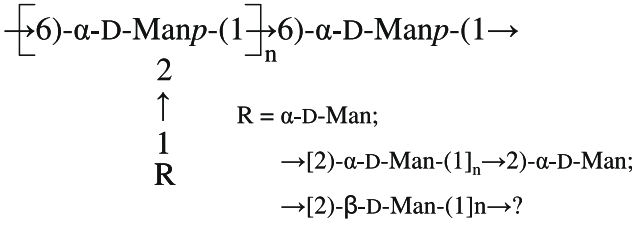
ARTICLE INFO

Article history:
Received 4 December 2009
Received in revised form 26 January 2010
Accepted 29 January 2010
Available online 16 February 2010

Keywords:
Cell-surface glycans
Helicobacter pylori
Lipopolysaccharide
Mannan
Vaccine

ABSTRACT

With the intent of contributing to a carbohydrate-based vaccine against the gastroduodenal pathogen, *Helicobacter pylori*, we report here the structure of cell-surface mannans obtained from a virulent strain. Unlike other wild-type strains, this strain was found to express in good quantities this polysaccharide in vitro. Structural analysis revealed a branched mannan formed by a backbone of α-(1→6)-linked mannopyranosyl residues with approximately 80% branching at the *O*-2 position. The branches were composed of *O*-2-linked Man residues in both α- and β-configurations:



In addition, this strain also expressed cell-surface emblematic *H. pylori* lipopolysaccharides (LPS) containing partially fucosylated polyLacNAc *O*-chains. Affinity assays with polymyxin-B and concanavalin A revealed no association between the mannan and the LPS. The described mannans may be implicated in the mediation of host–microbial interactions and immunological modulation.

1. Introduction

Helicobacter pylori is a widespread Gram-negative bacterium that colonizes the gastric mucosa of about 50% of the global human population.[1] *H. pylori* infection is currently recognized to be a crucial event in the induction of gastric malignancies such as gastritis, gastric atrophy and peptic and duodenal ulcers.[2] *H. pylori* is also directly correlated with an increased risk of developing gastric cancer,[3] indeed it is classified as a type I (definitive) human carcinogen.[4] So far there is no vaccine available against *H. pylori* and control of infection is accomplished by various combinations of antibiotic regimes,[5] a treatment that is gradually losing effectiveness due to the rise of antibiotic-resistant strains.[6] Based on the proven success of carbohydrate-based vaccines against several pathogenic bacteria,[7] much attention has been drawn towards the identification and structural characterization of *H. pylori* cell-surface glycans.

Like other Gram-negative bacteria, *H. pylori* expresses cell-surface lipopolysaccharides (LPS) that exert much influence in the mediation of host–pathogen interactions, namely, the adhesion to gastric cells and modulation of the host immune response.[8] It is now well established that *H. pylori* LPS comprises three distinct structural domains constituted by an elongated *O*-antigenic chain (*O*-chain), an oligosaccharide core and a fatty acid-rich endotoxin moiety, the lipid A (*O*-chain→core→lipid A ~ cell).[9] *H. pylori* *O*-chains are known to

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doi:10.1016/j.carres.2010.01.022

mimic glycosylation patterns in the gastric niche[10] by expressing structures analogous to some mammalian histo-blood-group antigens, namely, the Lewis (Le^a, Le^b, Le^x, sialyl Le^x, Le^y) and the blood groups A and B determinants.[9] *H. pylori* serotypes O:3 and O:6 further present an heptoglycan (D-glycero-α-D-manno-heptoses) domain between the Le *O*-chain and the core region (*O*-chain→heptoglycan→core→lipid A∼cell).[9] Also, some strains have shown elongated α-(1→6)-Glc chains linked to the core[11] or, as recently observed for *H. pylori* serotype O:2, a chain composed of alternating *O*-2- and *O*-3-linked α-D-glucopyranose residues [→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→]$_n$.[12]

In addition to the LPS, it has been recently demonstrated that *H. pylori* bioaccumulates considerable amounts of amylose-like glycans under specific environmental pressures such as subcultivation in solid medium.[13] The same study points out the existence of terminal, *O*-2-, *O*-6- and *O*-2,6-linked Man residues in the cell-surface glycan-rich extracts of *H. pylori* strains NCTC 11637 and clinical isolates 968 and 14255.[13] Furthermore, it was suggested that growing *H. pylori* in Ham's F12 liquid medium enhances the expression of Man.[13] Further suggesting the presence of cell-surface mannose-containing molecules, both *H. pylori* coccoid and spiral cellular forms have shown to agglutinate with the α-Man-specific lectin of *Pisum sativum*,[14] and coccoid cells have also demonstrated to agglutinate with the α-Man-specific lectins of *Lens culinaris* and *Narcissus pseudonarcissus*.[14] Despite these evidences, up to now only one report has been presented addressing the origin and structural organization of *H. pylori* cell-surface mannose-rich polysaccharides (mannans).[15] This polysaccharide was isolated from *H. pylori* mutant strain NCTC 11637 *pgm* having a knock-out of the phosphomannomutase gene and described as composed of trisaccharide-repeating blocks of terminal, *O*-2 and *O*-2,6 Man residues.[15] Silencing of phosphomannomutase was described as responsible for the absence of elongated LPS, thus facilitating the extraction and purification of external glycocalyx material regarded as yielding Man-rich polysaccharides.[15] In this work, it is reported for the first time the expression of mannans by an *H. pylori* wild-type strain, *H. pylori* 968, previously suspected of expressing this polysaccharide.[13]

2. Experimental

2.1. Bacterial cultures

H. pylori 968, isolated from a gastric biopsy of a patient diagnosed with a peptic ulcer, was provided by the Centre of Biological Engineering of the University of Minho (Braga, Portugal).

2.2. Definition of virulence profile

H. pylori virulence determinants cytotoxin-associated protein (*cagA*) and vacuolating cytotoxin (*vacA*) encoded by the *cagA* and *vacA* genes were accessed by polymerase chain reaction (PCR). PCR primers and conditions were as previously described.[16,17] PCR products were visualized by electrophoresis on 2% agarose gels.

2.3. Bacterial growth and purity assessment

H. pylori cells isolated from gastric biopsies by recovery to prewarmed Columbia blood Agar (CBA) (bioMérieux, France) and incubated at 37 °C for 72 h on a microaerophilic atmosphere 85% N_2, 10% CO_2 and 5% O_2). The cells were subcultivated after 48 h of incubation period with the conditions described above. The cells were also grown on Ham's F12-defined liquid medium without the addition of growth-enhancing factors. The cells were allowed to grow for 24 h in a glass flask under microaerophilic atmosphere under gentle stirring at 37 °C, as described by Testerman et al.[18] Purity assessment was assured by using a highly specific peptide nucleic acid probe in a fluorescence in situ hybridization procedure, according to the protocol described by Guimarães et al.[19]

2.4. Extraction and purification of cell-surface glycans

Cell-surface glycans were isolated from the cells using the hot phenol–water extraction, according to Westphal and Jann.[20] The aqueous layer was dialyzed against distilled water using a 1000 Da cut-off membrane and lyophilized. The resulting material was fractioned by gel permeation/adsorption chromatography (GPC) on a polyacrylamide Bio-Gel P-6 (Bio-Rad) (fractionation range: 1000–6000 Da) as described by Ferreira et al.[13]

2.5. Sugar composition and linkage analysis

Sugar composition analysis was performed by alditol acetate method as described by Harris et al.[21] The hydrolysis was done in 4 M trifluoroacetic acid (TFA) at 100 °C for 3 h, followed by reduction with $NaBH_4$ and subsequent acetylation with acetic anhydride in the presence of 1-methylimidazole. Alditol acetate derivatives were analyzed by gas chromatography–mass spectrometry (GC–MS), as described by Ferreira et al.[13] Linkage analysis was carried out by methylation with $NaOH/(CH_3)_2SO/CH_3I$, as described by Ciucanu and Kerek.[22]

2.6. Affinity chromatography using polymyxin-B

Mannose-rich material isolated in the void volume of the Bio-Gel P6 column was fractionated based on its affinity to polymyxin-B. Fractionation was performed according to Arafat et al.[23] in a polymyxin-B–agarose (Sigma Chemical Co.) column with 0.5 cm length and 0.25 cm diameter, operated at 5 °C with a constant flow rate of 0.2 mL min^{-1}. The resin was equilibrated with an endotoxin-free 0.1 M ammonium bicarbonate buffer, pH 8.0. Approximately 2.0 mg of sample was dissolved in the buffer to a final concentration of 0.5 mg mL^{-1} and centrifuged to remove the suspended particles and the resulting supernatant was loaded into the column. Non-retained material was recovered by elution with endotoxin-free buffer and the retained material was recovered by elution with 1% sodium deoxycholate in endotoxin-free buffer solution.

2.7. Affinity chromatography using a Concanavalin-A

Mannose-rich material isolated on the void volume of the Bio-Gel P6 column was fractionated based on its affinity to the concanavalin A lectin. Fractionation was performed on a concanavalin A (Con A) *from Canavalia ensiformis* (Jack Bean) agarose conjugate (Sigma) in a column with 0.5 cm length and 0.25 cm diameter, operated at 5 °C with a constant flow rate of 0.33 mL min^{-1}. Prior to elution the column was pre-washed with a solution composed of 1 M NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 5 mM $CaCl_2$, and equilibrated with a buffer solution of 20 mM Tris 0.5 M NaCl at pH 7.4. Approximately 2.0 mg of the initial sample was dissolved in the buffer to a final concentration of 1 mg mL^{-1}, centrifuged to remove the suspended particles and the resulting supernatant was loaded into the column. The non-retained material was recovered by elution with the buffer and the retained material was recovered in a stepwise elution using 5 mM, 25 mM and 100 mM of methyl-α-D-mannopyranoside in buffer solution.

2.8. NMR experiments

^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AMX 600 spectrometer equipped with a TXI probe at 310 K and a Varian

vnmrs 600 equipped with triple-resonance (^{1}H, ^{13}C, ^{15}N) cryoprobe and recorded at 310 K. Two-dimensional (2D) NMR correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY) and heteronuclear single quantum correlation (HSQC) spectroscopy were performed using the instrument's Bruker software. Prior to performing the NMR experiments, the samples were lyophilized three times with D_2O (99.9%). The HOD peak was used as the internal reference at δ_H 4.761 for ^{1}H NMR spectroscopy and orthophosphoric acid (δ_P 0.0) as the external reference for ^{31}P NMR experiments. Just before the NMR experiments were carried out, a D_2O sample containing TMS (δ_H 0.00) was run to aid referencing the HOD signal.

3. Results

H. pylori strain 968 recovered from a gastric biopsy specimen of a patient with peptic ulcer was characterized by expressing *cagA* and *vacA* (s1,m1), virulence markers that have been directly correlated with the promotion of gastric malignancies.[1]

3.1. Pre-characterization of *H. pylori* 968 glycan-rich water extracts

H. pylori 968 was grown both in CBA solid medium and in Ham's F12-defined liquid medium containing no Man. Growth in Ham's F12 liquid medium was carried out without the addition of growth-enhancing factors that could constitute external sources of polysaccharides. Cell-surface glycans were isolated in the aqueous phase resulting from hot phenol–water extraction performed on intact bacterial cells. The sugar composition of these extracts is presented in Figure 1 in terms of μg/mg of dry biomass and corresponds to the mean value from three independent growth batches. The results show that CBA glycan-rich extracts were composed mainly of LPS as observed by the considerable amounts of Gal (2.5 μg/mg), Glc (1.8 μg/mg), GalNAc (1.7 μg/mg) and lower content of DD-Hep (0.8 μg/mg), LD-Hep (0.7 μg/mg) and Fuc (0.4 μg/mg). Lower amounts (<1 μg/mg) of Rib, possibly from ribonucleic acids, and Man, as well as trace amounts of Rha and Ara were also present. Conversely, *H. pylori* growth in Ham's F12 liquid medium (Fig. 1) enhanced the expression of Man by five times in relation to CBA, suggesting the expression of mannans. The amount of Rha (1.5 μg/mg) and Rib (3.3 μg/mg) was also higher in F12. The amount of LPS sugars (Fuc, Glc, Gal, GlcNAc, DD-Hep and LD-Hep) was similar to the one observed in CBA, with the exception of Fuc (0.8 μg/mg) that doubled its concentration denoting an increase in the fucosylation of *O*-chains.

These results show that F12 liquid medium is more suitable for mannan expression than CBA. Furthermore, it overcomes contamination with Man from culture medium components present in CBA. The induction of growth without the addition of enhancing factors associated with *H. pylori* high fastidiousness resulted in very low biomass yields.

3.2. Isolation of a mannan-rich fraction

Linkage analysis (Table 1) showed that *H. pylori* 968 mannans recovered from F12 were built by mannopyranosyl residues consisting of terminal (10.3%), *O*-2-linked (12.0%), *O*-2,6-linked (9.0%) units and *O*-6-linked Man (4.3%). This material also contained *O*-4-linked Glc (9.6%) from amylose-like glycans[13] and *O*-3-linked Gal (10.1%) and *O*-4-linked GlcNAc (8.5%), recognized as LPS *O*-chain sugars.[9] The similar proportion of *O*-3-linked Gal and *O*-4-linked GlcNAc indicates *O*-chains exhibiting *N*-acetyllactosamine (LacNAc) moieties [→3)-Gal-(1→4)-GlcNAc-(1→]. Equal amounts of terminal Fuc (3.4%) and *O*-3,4-linked GlcNAc (4.0%) residues and the absence of branched Gal residues also suggested the expression of Type 1 Le^a and/or Type 2 Le^x blood group determinants.[9] The presence of LPS was further confirmed by the identification of low percentages of terminal and *O*-3-linked Glc, *O*-4-linked Gal, *O*-7-linked DD-Hep and *O*-2-, *O*-2,7- and *O*-3,7-linked ld Hep, normally observed in the core, and *O*-6-linked GlcNAc from lipid A.[9]

The Ham's F12 Man-rich preparation was subsequently eluted in a Bio-Gel P6 that removed the bulk of Rha and Rib residues. The majority of the material (~4.0 mg) was recovered in the void volume and was composed of 30% of sugars, 42% of which corresponding to Man residues.

Linkage analysis on this partially purified fraction (Table 1) revealed a slightly Man-enrichment mainly due to an increase in the percentage of *O*-2-linked Man. Conversely, the overall amounts of *O*-4-linked Glc decreased significantly, from 10.2% to 3.5%. The percentage of LPS-assigned sugars remained mostly unchanged when compared with the non-purified material.

3.3. NMR studies

The ^{1}H nuclear magnetic resonance (NMR) spectrum of the Man-rich fraction recovered from the P6 Bio-Gel column (Fig. 2) exhibited several resonances in the anomeric region δ_{1H} 4.9–5.4 ppm (Table 1). Additionally, ^{31}P NMR (data not shown) revealed the absence of any phosphorylation in these mannans.

Combined data from 2D ^{1}H–^{13}C HSQC experiments (Fig. 3), linkage analysis (Table 1) and available literature[24–27] resulted in the assignment of the resonances at $\delta_{1H/13C}$ 5.31/101.90 (residue A), 5.12/99.71 (residue B) and 5.05/103.20 (residue C), respectively, to *O*-2 Man, *O*-2,6 Man and terminal Man, all in α configuration.

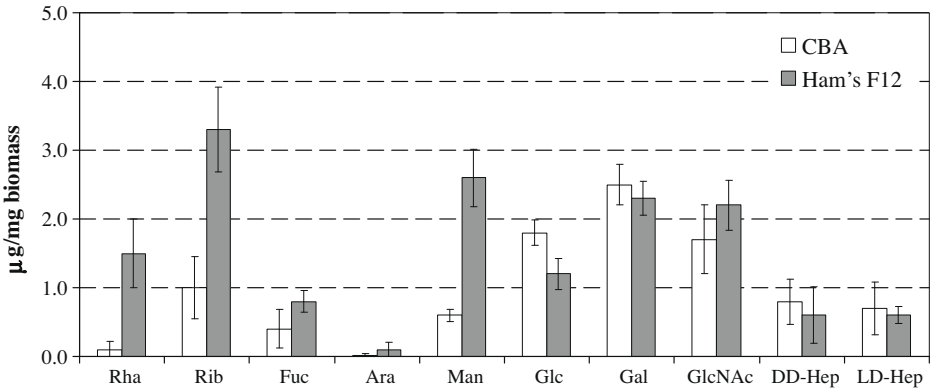

Figure 1. Sugar composition of *H. pylori* 968 glycan-rich water extracts resulting from growth in solid medium (CBA) and Ham's F12 liquid medium.

Table 1
Linkage profile of the sugars in glycan-rich extracts from the *H. pylori* 968 non-purified material and partially purified material recovered in the void volume of a Bio-Gel P6 column

Linkage type	Relative molar ratio (%)	
	Non-purified	Partially purified
LPS		
O-Chain		
T-Fuc	3.4	2.8
→6)-Glc	4.2	7.9
T-Gal	1.7	3.4
→3)-Gal	10.1	11.9
T-GlcNAc	1.8	2.5
→4)-GlcNAc	8.5	7.1
→3,4)-GlcNAc	4.0	3.1
Core		
*T-Glc	2.8	3.9
→3)-Glc	1.1	2.2
→4)-Gal	5.9	2.6
T-DD-*manno*-Hep	0.2	v
T-LD-*manno*-Hep	0.3	v
→2)-DD-*manno*-Hep	0.5	0.4
→2)-LD-*manno*-Hep	0.9	0.5
→3)-LD-*manno*-Hep	0.6	0.4
→7)-DD-*manno*-Hep	1.0	0.4
→7)-LD-*manno*-Hep	0.5	0.6
→3,7)-LD-*manno*-Hep	0.8	0.5
→2,7)-DD-*manno*-Hep	1.4	0.9
Lipid A		
→6)-GlcNAc	0.7	0.3
Mannose-rich glycans		
T-Man	10.3	10.8
→2)-Man	12.0	19.2
→6)-Man	4.3	3.8
→2,6)-Man	9.0	9.5
Amylose-like glycans		
→4)-Glc	9.6	3.5
→4,6)-Glc	0.6	–
Unassigned sugars		
→2)-Ara	0.6	–
→6)-Gal	3.2	2.8

* Terminal Glc residues can be addressed either to amylose-like polymers or to non-reducing ends of the LPS core. v—vestigial amounts; >0.5% total sugars.

A weak resonance at $\delta_{^1H\text{-}1}$ 4.88 (residue D in Table 2), resulting from vestigial amounts of α-*O*-6 Man residues (>5%, Table 1), in α configuration,[24] was also observed. Sequential assignment of proton signals for spin systems A–D was obtained from the 2D ^1H–^1H COSY (results not shown) and TOCSY (Fig. 4). The H-6 protons of *O*-2,6 Man residues have also been assigned from the HSQC spectrum (Table 2) based on the characteristic downfield shift of Man residues at $\delta_{^{13}C}$ 62.30–68.00 ppm upon substitution at the *O*-6 position.[24,27] The identified resonances are summarized in Table 2 and were found to be in accordance with the previous reports.[26] Additionally, NOESY experiments (Fig. 5) confirmed the α-configuration of spin system A assigned to *O*-2 Man residue by showing a medium intensity NOE (Nuclear Overhauser Effect) between the anomeric proton at 5.31 ppm and its H-2 at 4.12 ppm. Residue A also exhibited a low intensity inter NOE between its H-1 and the H-2 of residue B at 4.05 ppm demonstrating that *O*-2-linked Man are linked to the *O*-2 position of *O*-6 Man residues. It further revealed connectivity between the anomeric proton of residue B at 5.12 and its H-6 protons at 3.70 and 3.91 ppm, highlighting the presence of sequential *O*-6-linked B residues. The spin-system C corresponding to terminal Man residues showed a single medium intensity inner NOE between its anomeric proton at 5.05 ppm and the H-2 at 4.08 ppm confirming its α configuration. A NOESY spectrum acquired with a longer mixing time (data not shown), even though it resulted in strong signal overlapping, allowed the detection of a very weak connectivity between H-1 and the H-2 of residue A and of residue B. This observation led to the conclusion that *O*-2-linked α-Man side chains are terminated by α-Man residues and that some branches exhibit just one terminal residue. Combined information from NMR and linkage analysis thus suggest that *H. pylori* 968 expresses a mannan consisting of α-(1→6)-linked mannopyranose units with approximately 80% of *O*-2-substituted residues. Side chains were found to be composed of terminal and *O*-2-linked α-Man residues.

The integration of A–C anomeric resonances in the ^1H NMR spectrum retrieved a proportion of *O*-2, *O*-2,6 and terminal Man of approximately 1:1:1 suggesting the existence of a repetitive unit in this mannan. However, the proportion between α-Man residues calculated from the ^1H NMR spectrum revealed significantly lower amounts of *O*-2-linked Man residues than those shown by linkage analysis (Table 1). A residue E could also be assigned to the resonances at $\delta_{H1/1C}$ 4.92/101.25 ppm and showed in the TOCSY/HSQC spectra $\delta_{H2/C2}$ at 4.41/79.33 ppm and $\delta_{H3/C3}$ at 3.91/71.71 ppm. Furthermore, the NOESY spectrum (Fig. 5) exhibited a strong H-1/H-2 inner and inner NOE and a strong H-1/H-3 inner NOE. These

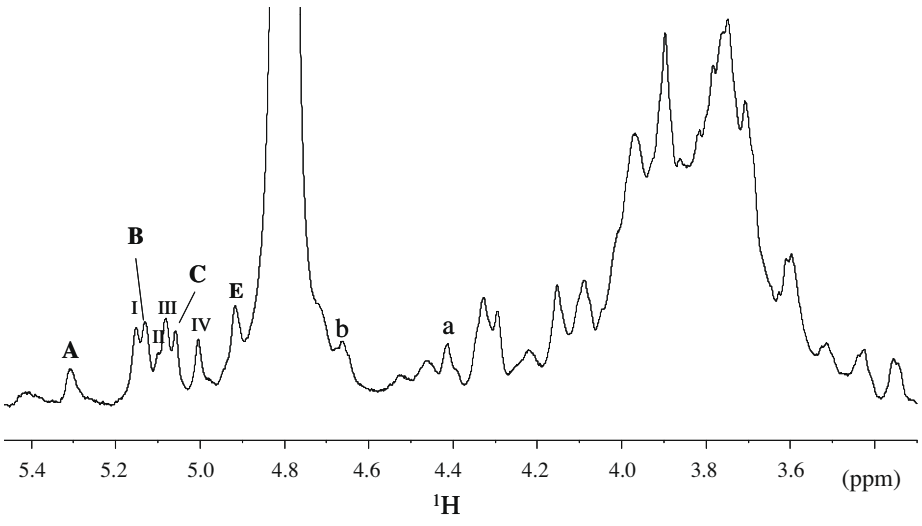

Figure 2. ^1H NMR spectrum of the Man-rich material recovered in the void volume of a P6 column.

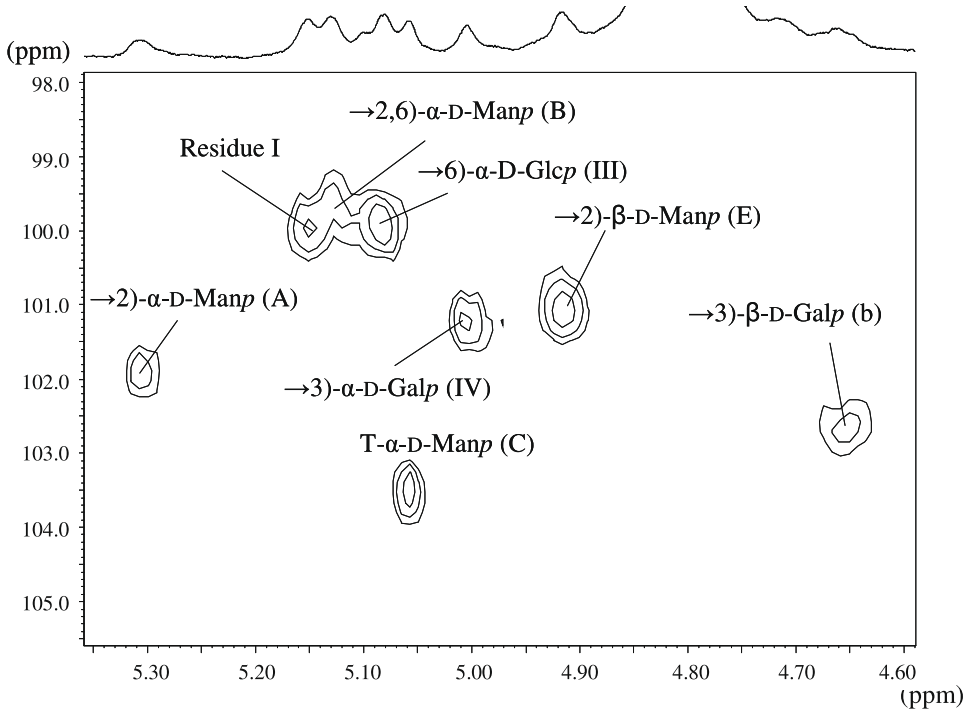

Figure 3. Anomeric region of the 2D ^{1}H–^{13}C HSQC spectrum of the Man-rich material recovered in the void volume of a P6 column.

Table 2
^{1}H NMR chemical shifts (ppm) from 2D COSY and 2D TOCSY and ^{13}C chemical shifts from HSQC spectra of Man-rich fraction

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	Assigned NOE anomeric region
LPS O-chain							
a	4.45	3.52	3.68	—	—	—	—
→3)-β-D-Gal	103.03	—	—				
b	4.65	3.84	—	—	—	—	—
→4)-β-D-GlcNAc	102.56	—					
Mannose moiety							
A	5.31	4.12	3.98	—	—	—	A1 (M); B2 (W)
→2)-α-D-Man	101.99	79.42	71.15				
B	5.12	4.05	3.71	3.91	—	3.91, 3.70	B2 (M); B6 (S)
→2,6)-α-D-Man	99.71	—	71.02	72.90		68.00	
C	5.05	4.08	3.85	—	—	3.90, 3.77	C2 (S); A2 (W)
T-α-D-Man	103.4	—	71.73			62.30	
D	4.88	3.98	—	—	—	—	—
→6)-α-D-Man	—	—					
E	4.92	4.41	3.91	—	—	—	D2 (S); D3 (S);
→2)-β-D-Man	101.20	79.33	71.70				B2 (VW)
Other structural motifs							
I	5.15	3.69	—	—	—	—	I2 (S); III6 (S)
Unassigned residue	99.99	70.70	—				
II	5.10	4.13	4.00	—	—	—	—
Unassigned residue	108.7	82.41	84.50				
III	5.08	3.36	3.62	—	—	3.92, 3.68	III2 (M); IV3
→6)-α-D-Glc	99.8	72.71	73.81	—		68.01	(VW)
IV	5.00	4.27	3.86	—	—	—	IV2 (S); IV3 (S)
→3)-α-D-Gal	101.1	70.78	74.20				

Legend: S—strong NOE; M—medium NOE; W—weak NOE; VW—very weak NOE.

assignments are consistent with the previous reports for inner chain *O*-2-linked Man residues in β configuration.[28,29] The NOESY spectrum of residue E highlighted the connectivity between its anomeric proton and the H-2 of residue B, revealing that some of the *O*-6 mannose side chains had *O*-2-linked β-Man residues.

The NMR studies also showed an array of low intensity signals from 5.50 to 5.34 ppm assigned to α-Hep from the LPS as well as *O*-3- and *O*-4-linked α-Glc residues and signals at 4.4–4.7 ppm assigned to *O*-3-linked Gal and *O*-4-linked GlcNAc residues in β configuration, characteristic of *O*-chains.[30]

Other resonances were observed in the α-anomeric region of the ^{1}H spectrum (Fig. 1), namely, at 5.15 ppm (residue I) exhibiting a carbon chemical shift of 99.95 ppm (Fig. 3). However, the anomeric region of the COSY and TOCSY (Fig. 2) spectra showed for this residue a single cross-peak with the H-2 proton at 3.69 ppm. The strong signal overlapping observed in the ring region

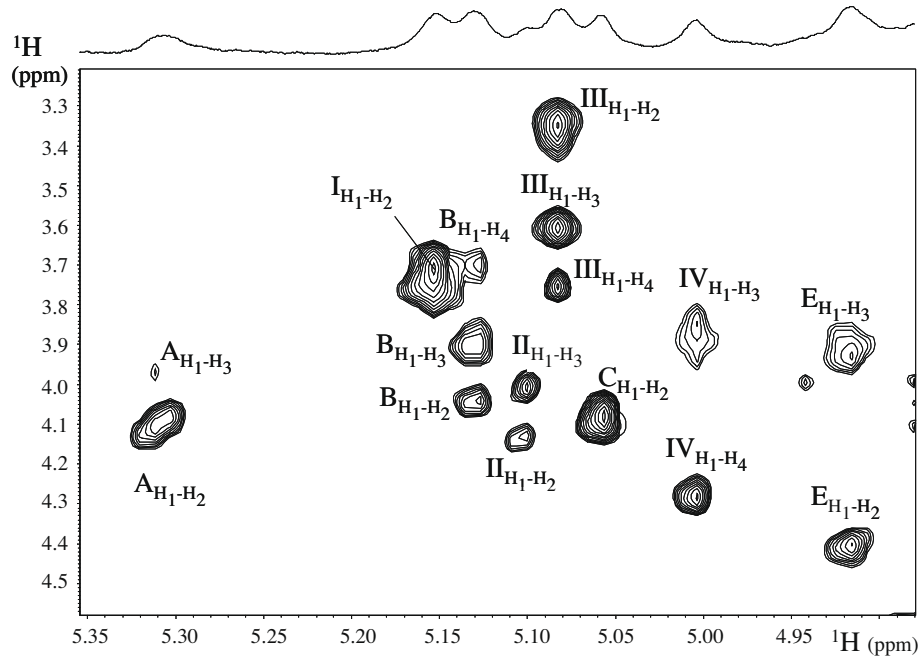

Figure 4. Anomeric region of the 2D ^{1}H–^{1}H TOCSY spectrum of the Man-rich material recovered in the void volume of a P6 column.

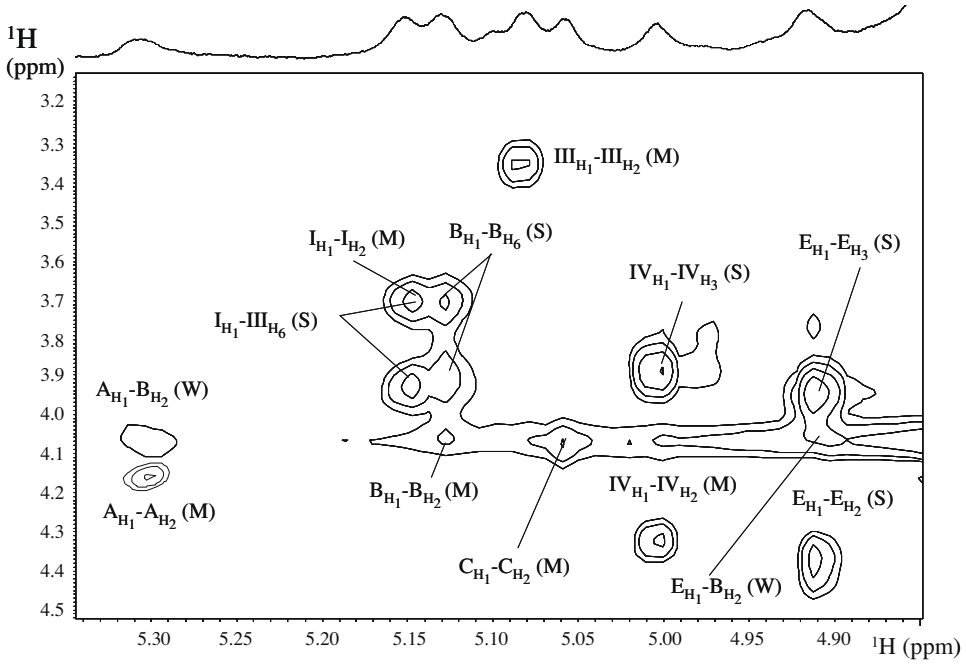

Figure 5. Anomeric region of the 2D ^{1}H–^{1}H NOESY spectrum of the Man-rich material recovered in the void volume of a P6 column.

prevented the identification of other inner-ring protons and the absence of other cross-peaks further compromised its identification. Another unassigned low intensity resonance was detected at 5.10 ppm (residue II in Table 2) and revealed a highly deshielded C1 chemical shift at 108.7 ppm (data not shown). Two additional strong resonances were detected at δ 5.19 ppm and 5.00 ppm and assigned, based on its anomeric chemical shifts and linkage analysis, to α-*O*-6-linked Glc (residue III) and α-*O*-3-linked Gal (residue IV), respectively. Still, the considerable shift upfield exhibited by the H-2 proton of residue *O*-6 in relation to a linear *O*-6-linked structure[31] should be pointed out. Additionally, the NOESY spectrum (Fig. 4) exhibited a cross-peak between the anomeric proton of residue I and H-6 protons of residue II at 3.92 ppm and 3.68 ppm confirming both the assignment of residue II to *O*-6 Glc and the connectivity between the two residues. It also showed a weak intra-residue NOE between the H-1 of residue II and the H-3 of residue III (Table 2) therefore confirming the assignment of residue III to *O*-3 Gal and revealing that residue II is linked to the *O*-3 position of residue III. In addition, the NOESY also highlighted the existence of contiguous *O*-3-linked α-Gal residues by showing a strong H-1/H-3 NOE at 5.00/3.85 ppm in addition to the H-1/H-2 inner NOE at 5.00/4.27 ppm (Fig. 5). Interestingly, the integration of the anomeric protons of residues I, II and III in the ^{1}H NMR spectrum (Fig. 2) revealed a proportion of

1.0:1.0:1.5, thus consistent with the observation of sequential *O*-3-linked α-Gal residues, also seen by linkage analysis (Table 2). These observations thus suggest the following structural motif: α-residue I-(1→6)-α-D-Glc-(1→3)-α-D-Gal-(1→3)-α-D-Gal-(1→. The experimental proton and carbon chemical shifts recovered from the COSY, TOCSY and HSQC spectra for residues II and III (Table 2) matched the theoretical simulation for the oligosaccharide sequence →6)-α-D-Glc-(1→3)-α-D-Gal-(1→3)-α-D-Gal-(1→ using CASPER.[28] This calculation revealed a low overall error of 6.38 ppm between the predicted and experimental data further confirming the mentioned assignment. However, the NOESY spectrum revealed no connectivity between the described structural motif and the mannan moiety or LPS-related signals.

3.4. Evaluation of mannan-LPS associations

Despite being mainly composed of Man residues, the partially purified material isolated in the void volume of the Bio-Gel P6 column also exhibited LPS in its composition. To evaluate the possible associations between these two domains this fraction, referred as starting material in Table 3, was fractionated by affinity chromatography.

3.4.1. Purification of mannose-rich glycans using polymyxin-B

The Man-rich material was fractionated according to its ability to bind to the amphiphilic polypeptide polymyxin-B, an antibiotic recognized for its affinity for LPS.[32] The non-retained fraction (Table 3) that corresponded to approximately half of the initial material lacked LPS as shown by the absence of Fuc, DD-Hep and LD-Hep residues. This fraction was composed of 28% of sugars, from which approximately 50% was Man, and also of lower percentages of Glc (18%), Gal (17%) and GlcNAc (5%). The isolation of mannans in an LPS-free fraction suggested that there was no covalent linkage between these two domains. Conversely, the material recovered from the column using sodium deoxycholate (Table 3) was mainly composed of LPS (22% Gal, 23% GlcNAc and ~4% of Fuc, DD- and LD-Hep). However, both the 1% and 5% sodium deoxycholate eluted material also exhibited Man (16–27%). Thus, even though the bulk of the mannans revealed no affinity to polymyxin-B, a lower percentage showed affinity to polymyxin-B denoting some amphiphilic behaviour.

3.4.2. Purification of mannose-rich glycans using concanavalin A

The Man-rich material was also fractionated according to its affinity to concanavalin A, a lectin recognized to bind to terminal α-D-mannosyl and α-D-glucosyl[33] residues. The non-retained material was found to be sugar-poor (7% of sugars) and almost exclusively composed of residual amounts of ribose. The retained molecules were further recovered in a stepwise elution using 5 mM, 25 mM and 100 mM methyl-α-D-mannopyranoside. The first fraction yielding 27% of the initial material showed a low abundance of sugars (17%), mainly Man-rich material (39%) but also LPS (25% Gal, 9% GlcNAc, 4% Fuc, 3% LD-Hep and 2% DD-Hep) and amylose-like glycans (18% Glc). Conversely, the 25 mM methyl-α-D-mannopyranoside fraction exhibited the highest sugar content (45%), 55% of which corresponded to mannans. This fraction lacked LPS, as revealed by the absence of Fuc and core-characteristic Hep residues. Still, it contained 21% Glc, 17% Gal and 7% GlcNAc. The identification of a Man-rich LPS-free fraction further reinforced the observation made upon polymyxin-B for the lack of association between these cell-surface glycans. Interestingly, in both cases, LPS-free mannan-rich material also contained Glc, Gal and GlcNAc. The results suggest that these sugars might be a part of the mannan moiety and further structural studies need to be conducted to confirm this observation.

The remaining material was recovered with 100 mM methyl-α-D-mannopyranoside and resulted in a fraction containing 32% of sugars, mainly LPS yielding a high degree of fucosylation (9% of Fuc). This fraction also contained mannans, even though in much lower percentage than the previous ones (9%).

3.4.3. Evaluation of linkage pattern of isolated mannans

The linkage pattern of the mannans recovered with 5 mM methyl-α-mannopyranoside from the concanavalin A column (Table 4) confirmed a mixture of Man-rich material, LPS and amylose-like glycans previously suggested by sugar analysis (Table 3). The mannan moiety in this fraction exhibited a mean percentage of backbone branching of 70%, thus lower than that observed in the starting material (80%, Table 4). It also showed a much lower percentage of *O*-2-linked Man side chain sugars (8.0%) in relation to branched residues (11.5% of *O*-2,6 Man), revealing that some of the mannan branches contain a single Man residue. Conversely, the mannans in the 25 mM LPS-free fraction had higher branching (~80%) as a result of an increase in the percentage of *O*-2,6 Man residues (~22%). Furthermore, they exhibited a lower proportion of *O*-2: *O*-2,6-linked Man (1:2). Additionally, 7–10% of terminal and *O*-6-linked Glc and *O*-3 Gal and lower percentages (<5%) of *O*-3 and *O*-4 Glc, terminal and *O*-6-linked Gal and terminal and O-4-linked GlcNAc were also observed. Linkage analysis of the fraction recovered with 100 mM methyl-α-mannopyranoside revealed LPS exhibiting over 40% of the core material. The *O*-chains were composed of LacNAc units (17.3% of *O*-3 Gal and 8.9% of *O*-4 GlcNAc) and Le blood group determinants as shown by the detection

Table 3
Sugar analysis and protein content of the material resulting from fractionation of the *H. pylori* 968 Man-rich on polymyxin-B and concanavalin A columns

Fraction	Yield (%)	Molar ratio (%)									Total sugars (%)
		Rha	Rib	Fuc	Ara	Man	Glc	Gal	GlcNAc	DD-Hep	LD-Hep	
Starting material	—	1	6	3	1	42	16	17	11	2	4	29
Polymyxin-B												
Non-retained	49	—	14	—	—	46	18	17	5	—	—	28
Retained												
1% Sodium deoxycholate	30	—	10	4	—	27	13	20	22	2	2	35
5% Sodium deoxycholate	4	—	3	3	—	16	23	22	22	5	6	7
Concanavalin A												
Non-retained	39	1	60	2	2	11	2	13	9	1	1	7
Retained												
5 mM Methyl-mannopyranoside	27	—	—	4	—	39	18	25	9	2	3	17
25 mM Methyl-mannopyranoside	19	—	—	—	—	55	21	17	7	v	v	45
100 mM Methyl-mannopyranoside	17	—	—	9	—	9	12	22	28	9	11	32

v—vestigial amounts; >0.5% total sugars.

Table 4
Linkage profile of the sugars observed in *H. pylori* 968 fractions showing affinity to concanavalin A

Linkage type	Relative molar ratio (%) [Methyl-α-D-mannopyranoside]		
	5 mM	25 mM	100 mM
LPS			
O-chain			
T-Fuc	3.0	—	7.0
→6)-Glc	9.0	—	2.4
T-Gal	1.9	—	3.5
→3)-Gal	10.7	—	17.3
T-GlcNAc	0.3	—	4.5
→4)-GlcNAc	5.0	—	8.9
→3,4)-GlcNAc	1.5	—	5.4
Core			
*T-Glc	6.2	—	5.8
→3)-Glc	1.1	—	4.6
→4)-Gal	6.3	—	4.8
T-DD-*manno*-Hep	v	—	0.7
T-LD-*manno*-Hep	v	—	0.9
→2)-DD-*manno*-Hep	0.3	—	1.2
→2)-LD-*manno*-Hep	0.9	—	6.0
→3)-LD-*manno*-Hep	0.6	—	2.8
→7)-DD-*manno*-Hep	0.9	—	3.9
→7)-LD-*manno*-Hep	0.8	—	2.9
→3,7)-LD-*manno*-Hep	0.8	—	2.2
→2,7)-DD-*manno*-Hep	1.2	—	2.5
Lipid A			
→6)-GlcNAc	1.0	—	—
Mannose-rich glycans			
T-Man	11.4	22.4	1.4
→2)-Man	8.0	10.2	10.9
→6)-Man	4.8	5.1	—
→2,6)-Man	11.5	21.6	1.2
Amylose-like glycans			
T-Glc*	—	9.1	—
→4)-Glc	8.1	4.2	0.3
→4,6)-Glc	—	—	—
Unassigned sugars			
→3)-Glc	—	2.5	—
→6)-Glc	—	6.8	—
T-Gal		2.3	
→4)-Gal	—	0.8	—
→3)-Gal	—	10.1	—
→6)-Gal	4.7	2.0	—
T-GlcNAc	—	1.5	—
→4)-GlcNAc	—	1.4	—

* Terminal Glc residues can be addressed either to amylose-like polymers or to non-reducing ends of the LPS core. v—vestigial amounts; >0.5% total sugars.

of terminal Fuc (7.0%) and O-3,4-linked GlcNAc residues (5.4%). Contrasting with other fractions, the mannan moiety in this one did not exceed 15% of the total sugars and was mainly composed of *O*-2-linked Man (~80% of the total Man). Furthermore, no *O*-6-linked residues were detected revealing a totally substituted polysaccharide with elongated side chains.

The structural diversity observed for the mannan moiety throughout the three studied fractions revealed the absence of a structure formed by repeating blocks but rather a random organization showing different branching degrees and side chain extensions and even substitution.

4. Discussion

The expression of vestigial amounts of Man at *H. pylori* cell surface has been long postulated[14,34] and found widespread among several strains.[13,14,34] Furthermore, we have observed that some fresh clinical isolates express high amounts of Man. Still, after very few subcultivations in vitro, Man expression decreased to residual levels (<5% of the cell-surface sugars). As a result of its low biological abundance structural assignment of *H. pylori* cell-surface Man has remained a difficult task. However, the previous studies have highlighted that *H. pylori* wild-type virulent strain 968 over-expressed Man, even after successive subcultivation in CBA.[13]

In an attempt to further potentiate the expression of mannans, this strain was grown in Ham's F12 liquid medium, known by its capability to overcome *H. pylori* high fastidiousness.[35] *H. pylori* cells recovered from F12 had an enhanced mannan expression when compared with the ones recovered from solid medium. This was most likely a result of pH decrease from 6.5 to approximately 5.0, a consequence of continuous sparging of the medium with CO_2 during growth. Such pH variations have been described to exert strong influence in gene expression and modulation of the activity of the enzymes related with the biosynthesis of GDP-D-Man.[36,37] In *H. pylori*, GDP-D-Man is mainly produced from D-fructose-6-phosphate in a three-step pathway[36,38,39] and the first and third steps are catalyzed by a bifunctional phosphomannose isomerase/GDP-D-mannose phosphorylase, an enzyme found only in bacteria and involved in the expression of capsular polysaccharides.[38] GDP-D-Man is the main precursor of GDP-L-Fuc used by *H. pylori* in the fucosylation of LPS *O*-chains[39] using a pathway with three additional steps.[39] A decrease in pH from 6.5 to 5.0 has been recognized to enhance the expression of the *wbcJ* gene, encoding a protein homologue involved in the conversion of GDP-Man to GDP-Fuc.[36] Thus, pH conditions encountered in *H. pylori* 968 culture medium may be considered to favour the fucosylation of LPS *O*-chains. However, the enzymes involved in the conversion of GDP-D-Man into GDP-L-Fuc have been described to have optimal pH activities at 6.5 and 8.0, respectively,[37] contributing to an increase in the pool of GDP-D-Man residues available for the production of Man-rich glycans. It should also be pointed out that GDP-D-Man is also the precursor of other activated nucleotides, namely, GDP-6-deoxy-D-talose and GDP-D-Rha,[39] thus explaining the presence of Rha in glycan-rich water extracts.

The mannans at *H. pylori* 968 cell surface were found co-expressed with LPS with *O*-chains carrying LacNAc units and Le blood group determinants. NMR structural studies demonstrated that *H. pylori* 968 mannans are formed by a sugar backbone of α-(1→6)-linked mannopyranose units exhibiting an average branching of 80% at the *O*-2 position and side chains composed of *O*-2-linked residues. Interestingly, the *O*-2 side chain residues were found to be either in α- or in β-anomeric configuration. Similar mannans have been observed in cell surface of several other microorganisms, namely, in the cell wall of yeasts,[26,29,40] and fungi,[25] or in the form of phosphorylated mannans and arabinomannans in mycobacteria.[41] They have further been detected at the cell surface of Gram-positive bacteria,[27,42] as well as in exopolysaccharides from Gram-negative *Pseudomonas syringae* pv. *ciccaronei*[24] and *Porphyromonas gingivalis*.[43] However, some structural variability was observed mainly in the percentage of branching of the backbone and substitution pattern of its side chains. Mannan side chains similar to the ones of *H. pylori* 968, exhibiting both terminal and/or *O*-2-linked Man in α and/or β configurations have also been described in yeasts[29] and Gram-positive[27] bacteria. Moreover, the microorganism mannans may present *O*-3-linked Man, terminal Glc,[24] and α-Ara[27,41] as side chains. The structural diversity observed may be thought to also occur in *H. pylori* 968 where considerable amounts of unassigned terminal and *O*-6-Glc and *O*-3-Gal were systematically found in Man-rich extracts.

Alpha mannans were also found as *O*-chains in the LPS of Gram-negative enterobacteria *Hafnia alvei* strain PCM 1223,[44] *Citrobacter braakii*,[45] *Escherichia coli* serotype O8,[46] and O9[47] and *Klebsiella* pneumoniae serotype O5.[46] However, in the mentioned cases, the mannan backbone was formed by *O*-2- and *O*-3-linked Man residues whereas in *H. pylori* 968 the backbone is formed by *O*-6- and *O*-2,6-linked Man.

H. pylori cell-surface mannans seem to be directly involved in the mediation of host–microbial interactions and immunological modulation. In agreement with these considerations, the data have been presented that *H. pylori* cells can interact with the mannose-binding lectin (MBL) protein,[48] coded by the *MBL-2* human gene, a key component in systemic and mucosal innate immunity.[49–51] MBL was found to induce bacterial elimination[52,53] by activating the complement system and to promote complement-independent opsonophagocytosis.[54] Besides activating local immune responses against the bacteria, the interaction between *H. pylori* mannans and the MBL protein may also exert much influence in the clinical outcome resulting from infection.

H. pylori mannans may also have an antigenic potential resulting from their capability to trigger immune responses[48] making them potential candidates to include in a carbohydrate-based vaccine. Furthermore, they overcome some of the limitations shown by other glycans found in *H. pylori* cell surface, namely, amylose-like polysaccharides widespread through nature and LPS *O*-chains recognized to express mammalian Lewis histo-blood group determinants.

Acknowledgements

This work was supported by Fundação para a Ciência e Tecnologia (FCT) through project Pylori E&LPS POCI/QUI/56393/2004, PhD grant SFRH/BD/19929/2004, by the Natural Sciences and Engineering Research Council of Canada (NSERC), and by the European Network of Research Infrastructures within the 6th Framework Programme of the EC (Contract # RII3-026145, EU-NMR). The authors further thank Dr. Adrien Favier (RALF-NMR facility, Grenoble – France) for conducting NMR experiments.

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