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Helicobacter pylori lipopolysaccharide structural domains and their recognition by immune proteins revealed with carbohydrate microarrays

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ABSTRACT

The structural diversity of the lipopolysaccharides (LPSs) from *Helicobacter pylori* poses a challenge to establish accurate and strain-specific structure-function relationships in interactions with the host. Here, LPS structural domains from five clinical isolates were obtained and compared with the reference strain 26695. This was achieved combining information from structural analysis (GC-MS and ESI-MS^n) with binding data after interrogation of a LPS-derived carbohydrate microarray with sequence-specific proteins. All LPSs expressed $Lewis^{x/y}$ and *N*-acetyllactosamine determinants. Ribans were also detected in LPSs from all clinical isolates, allowing their distinction from the 26695 LPS. There was evidence for 1,3-D-galactans and blood group H-type 2 sequences in two of the clinical isolates, the latter not yet described for *H. pylori* LPS. Furthermore, carbohydrate microarray analyses showed a strain-associated LPS recognition by the immune lectins DC-SIGN and galectin-3 and revealed distinctive LPS binding patterns by IgG antibodies in the serum from *H. pylori*-infected patients.

1. Introduction

Helicobacter pylori is a pathogenic Gram-negative bacterium that colonizes the human stomach of about 50 % of world's population (Hooi et al., 2017). Persistent infections often lead to several gastric diseases, ranging from chronic gastritis to gastric carcinoma (Parreira, Duarte, Reis, & Martins, 2016). It is well established that carbohydrates (glycans) play a pivotal role in host-*H. pylori* interactions. On the one hand, the adherence of bacteria to the gastric mucosa, which promotes initial attachment and persistence, is mediated by bacterial adhesins that target host glycans (Magalhães & Reis, 2010). On the other hand, lipopolysaccharides (LPSs), the major components of the bacterial outer membrane, act as pathogen-associated molecular patterns for pathogen recognition receptors expressed on host cells, with the ability to modulate and to trigger immune responses (Müller, Oertli, & Arnold, 2011).

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The structure of LPS encompasses a lipid A and a core oligosaccharide (OS) domain, which are conserved within a species, and a long chain repeat-unit polysaccharide termed O-antigen (O-chain), with a remarkable structural diversity. LPSs can occur with or without O-antigen chains, being termed smooth- or rough-LPSs, respectively (Ferreira, Silva, Monteiro, & Coimbra, 2012). A unique feature of the O-chain of the LPSs of most *H. pylori* strains is the expression of Lewis (Le) antigens, mimicking those of gastric epithelium (Monteiro, 2001). This camouflage assists bacteria to escape from host's immune responses and may also induce autoimmune disorders, contributing to the severity and chronicity of infection (Moran & Prendergast, 2001).

Earlier structural and immunological studies established that the core OS of *H. pylori* LPS comprises an inner and outer core (Ferreira et al., 2012). More recently, a redefinition of *H. pylori* LPS has proposed (*i*) the core OS as a short conserved hexasaccharide without the canonical inner and outer core architecture; and (*ii*) the O-antigen composed of epitopes previously assigned to the outer core OS, namely a conserved trisaccharide (Trio), the variable glucan and heptan, identified in many *H. pylori* strains, followed by the well-established *N*-acetyllactosamine (LacNAc) and Le antigens (Fig. 1) (Li et al., 2017). Other glycan components, such as ribans (Altman, Chandan, Li, & Vinogradov, 2011), mannans (Ferreira, Azevedo et al., 2010) and amylose-like glycans (Ferreira et al., 2009) have also been identified for some *H. pylori* strains.

This notable structural diversity of *H. pylori* LPS often hampers the establishment of accurate and strain-specific structure-function relationships in interactions with the host. Thus, structural studies of LPS from different *H. pylori* strains, as well as their recognition by host proteins, would contribute to a better understanding of their molecular complexity and role in pathogenesis.

Microarrays of sequence-defined glycans are powerful tools to address the specificity of carbohydrate recognition systems and elucidate carbohydrate structures involved both in endogenous biological processes and interactions with microbes. Since the first proof-of-concept studies (Blixt et al., 2004; Fukui, Feizi, Galustian, Lawson, & Chai, 2002; Wang, Liu, Trummer, Deng, & Wang, 2002), various microarray platforms have been constructed and different glycan libraries assembled with the aim of answering different biological questions (Li et al., 2018; Manimala, Roach, Li, & Gildersleeve, 2006; Palma et al., 2015; Song et al., 2011; Wu et al., 2019; Zong et al., 2017). Although most of the glycan probe libraries are of mammalian type, the glycan repertoire is increasing in number and diversity, particularly to cover microbe-derived structures, in the form of sequence-defined bacterial fragments (Geissner et al., 2019; Stowell et al., 2014), as well as bacterial PS (Stowell et al., 2014; Wang et al., 2002) or intact LPS (Stowell et al., 2014; Thirumalapura, Morton, Ramachandran, & Malayer, 2005). The value of these microbial glycan-focused arrays has been demonstrated in examining host responses to pathogenic bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Salmonella typhimurium* and *Shigella flexneri*, among others (Geissner et al., 2019; Stowell et al., 2014; Thirumalapura et al., 2005). However, to the best of our knowledge, none of these arrays have covered glycan fragments or intact LPSs from *H. pylori* strains.

The present work has aimed to explore the structural diversity of LPSs from five different *H. pylori* clinical isolates associated with gastric pathologies, in comparison with the reference *H. pylori* strain 26695. To achieve this, a microarray of *H. pylori* LPSs and other bacterial LPSs was constructed for their interrogation with carbohydrate sequence-specific proteins to complement the information derived from GC-MS and electrospray (ESI) mass spectrometry (MS) and tandem mass spectrometry (ESI-MS^n) on the different LPS structural domains. The microarray generated was also used as a tool for recognition of *H. pylori* LPSs by host immune receptors and for serum antibody profiling against these polysaccharides in *H. pylori*-infected cases.

2. Material and methods

An overview of the strategy used in this work is in supplementary scheme 1.

2.1. H. pylori strains and culture conditions

Five *H. pylori* clinical isolates (14255, 14382, CI-117, 2191 and CI-5), associated with gastric conditions (peptic ulcer, reflux esophagitis, dyspepsia and chronic gastritis), and the reference strain 26695 (ATCC 700392), were used in this study. *H. pylori* cells were recovered from frozen stock cultures (Brucella Broth with 20 % glycerol), plated onto Columbia agar media supplemented with 5 % (v/v) horse blood (bioMérieux) and incubated at 37 °C for 72 h on a microaerobic atmosphere generated by GasPak EZ Campy Container System Sachets (BD). Cells were sub-cultivated after 48 h incubation periods in the conditions described above. The biomass was harvested to sterile phosphate buffer saline for subsequent extraction of their LPS. Culture purity was assessed by cellular morphology (curved Gram-negative bacilli) after Gram staining and by positive biochemical reactions for oxidase, catalase and urease tests (Table S1), following established procedures (Zhang et al., 2015).

2.2. Human sera

Serum samples were collected at Centro Hospitalar do Porto (Portugal) using standard procedures. All cases and controls underwent white light upper endoscopy and biopsies from antrum and corpus were obtained for histopathological study and *H. pylori* culture (Marcos-Pinto et al., 2012). A total of 24 sera were used in this study: 12 from individuals Hp+ with moderate/severe inflammation and 12 from individuals Hp- with absent/mild inflammation. Clinical features regarding the analyzed Hp + and Hp- cases are in Table S2. The age of the infected individuals varied from 32 to 62, with an average value of 47 years, and included 7 males and 5 females. For Hp- controls, the age of individuals varied from 18 to 59 with an average value of 39 years and included 5 males and 7 females. The criteria for selection of Hp + and Hp- control sera were based on the histology and culture tests (positive or negative for *H. pylori*), levels of serum *H. pylori* IgG/IgA antibodies detected with the anti-*H. pylori* ELISA commercial kit (EUROIMMUN Medizinische Labordiagnostika AG), degree of inflammation and infiltration of polymorphonuclear cells (PMN infiltration), observation of atrophy or intestinal metaplasia (Table S2).

2.3. Extraction and fractionation of H. pylori LPSs

H. pylori LPSs were extracted from 1−2 g of bacteria by the hot phenol-water method (Westphal & Jann, 1965). The LPS-enriched aqueous layer was recovered, dialyzed (membrane cut-off 1 kDa) and lyophilized. The resulting material (19−58 mg dry weight) was resuspended in 50 mM Tris-HCl (pH 7.5) containing 6 mM $MgCl_2$ and 2 mM $CaCl_2$ and subject to enzymatic treatments with DNAse, followed by RNAse, each overnight at 37 °C, and by proteinase K, overnight at 45 °C, to remove nucleic acids and protein contaminants, respectively. DNAse (EN0521), RNAse (EN0531) and proteinase K (EO0491) were from Thermo Fisher Scientific.

LPSs were fractionated by gel filtration on a Sephacryl S-300 HR (GE-Healthcare, 2−400 kDa) column (0.77 m length and 1.6 cm diameter), previously calibrated with blue dextran 2 MDa, dextrans 150 kDa and 12 kDa, and Glc (all from Sigma), and the average molecular weights (Mw) estimated (Fig. S1). The samples were eluted with 3 M urea in 0.1 M phosphate buffer (pH 6.5) at a flow rate of 0.5 mL/min, and 1 mL fractions were collected. Absorbance at 260 and 280 nm was used to monitor nucleic acids and protein contents, respectively. Total monosaccharide content was determined by the colorimetric phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1951) and

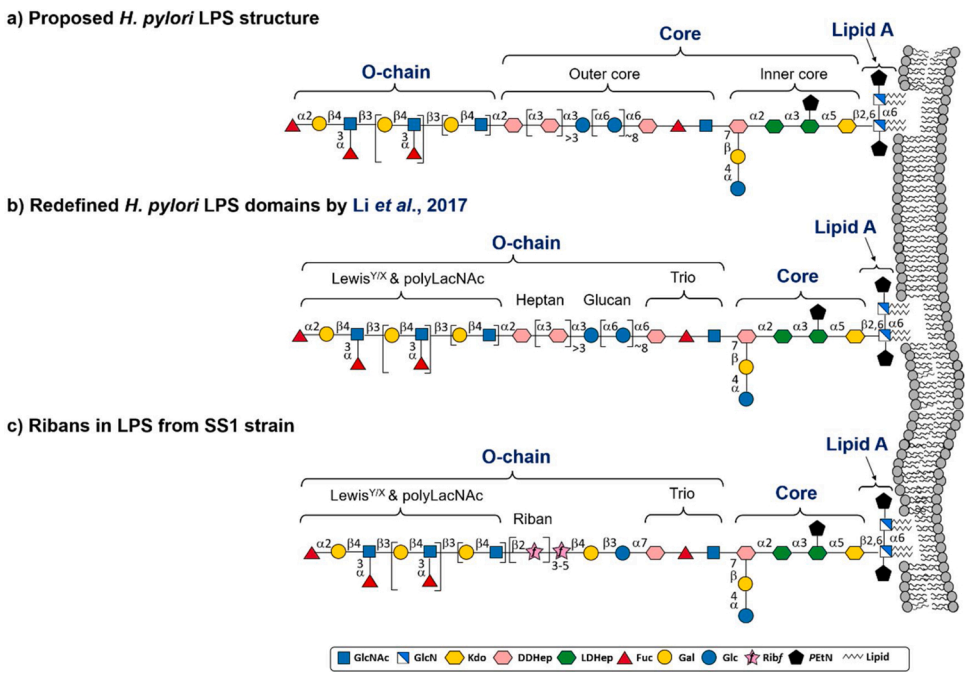


Fig. 1. General architecture of *H. pylori* LPS based on (Li et al., 2017) and adapted from (Ferreira et al., 2012). **a)** Previously proposed LPS domains structure of *H. pylori* 26695 with the O-antigen containing Lewis $(Le)^{y/x}$ determinants and *N*-acetyllactosamine (LacNAc) backbones, and the inner and outer core comprising the conserved hexasaccharide, and the Trio, glucan and heptan, respectively; **b)** redefinition of the LPS domains with the O-chain encompassing the epitopes previously assigned to the outer core OS (Trio, glucan and heptan) and the well-established LacNAc and Le determinants; **c)** The LPS of *H. pylori* SS1 strain containing ribans (Altman et al., 2011b). The representation of glycans follows the guidelines of Symbol Nomenclature for Glycans (Neelamegham et al., 2019). Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; DD-Hep, D-*glycero*-D-*manno*-heptose; LD-Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy--D-*manno*-octulosonic acid; *P*EtN, phosphoethanolamine; Rib*f*, ribofuranose.

expressed as equivalents of glucose. The fractions rich in carbohydrates, corresponding to LPS fractions, were dialyzed, lyophilized and stored in a desiccator at room temperature until further analysis.

H. pylori LPSs (10–50 μg) from S-300 fractions were separated by SDS-PAGE as described (Laemmli, 1970) with some modifications. Briefly, the lyophilized *H. pylori* LPSs were dissolved in the Tris (hydroxymethyl)aminomethane (Tris)-based sample buffer (NP0008, Invitrogen**)** and heated for 10 min at 100 °C. *H. pylori* LPS samples (10–20 μL) and a LPS molecular marker (250 μg/mL, P-20495, Molecular Probes) were run on a Bis-Tris mini precast 1 mm polyacrylamide gel of 4–12 % acrylamide gradient (Invitrogen) at 35 mA in the 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer (Invitrogen) for 90 min. The gel was fixed with 50 % (v/v) ethanol and 10 % (v/v) acetic acid in ultrapure water, for 60 min, and visualized using a commercially available silver staining kit (PROTSIL1-1KT, Sigma) (Tsai & Frasch, 1982).

2.4. *H. pylori* LPS-derived oligosaccharides

Aiming to obtain representative oligosaccharide structures present in the clinical isolates that differ from the reference strain, LPSs from CI-5 and 26695 were selected and subjected to partial acid hydrolysis with 50 mM trifluoracetic acid (TFA) for 1 h at 65 °C. The TFA was evaporated at room temperature, and the resulting mixture was re-suspended in distilled water and fractionated by gel filtration on a polyacrylamide Bio-Gel P2 (Bio-Rad, 1.0 m length and 1.0 cm diameter), at a constant flow of 0.5 mL/min. Fractions of 1 mL were collected and assayed for total sugars using the phenol-sulfuric acid method as above.

2.5. Glycosidic linkage analysis

The composition of *H. pylori* LPSs and LPS-derived oligosaccharides (80 μg total sugars) was determined by methylation analysis following the $NaOH/Me_2SO/CH_3I$ method (Ciucanu & Kerek, 1984). The methylated polysaccharides were hydrolyzed with 2 M TFA at 121 °C for 60 min, reduced with $NaBD_4$, and acetylated with acetic anhydride in the presence of 1-methylimidazole (Coimbra, Delgadillo, Waldron, & Selvendran, 1996; Harris, Henry, Blakeney, & Stone, 1984). The partially methylated alditol acetates were dissolved in anhydrous acetone and analyzed by GC-MS (Agilent Technologies 6890 N Network Gas Chromatography connected to an Agilent 5973 Selected Mass Detector). The GC was equipped with a DB-1 ms capillary column (100 % dimethylpolysiloxane, 30 m length, 0.25 mm internal diameter, and 0.10 μm film thickness) (122−0131, Agilent). The samples were injected in splitless mode (time of splitless 1.00 min), with the injector and detector operating at 230 °C, using the following temperature program: 50 °C (9 min) → 140 °C (5 min) at 10 °C/minute → 170 °C (1 min) at 0.5 °C/min → 230 °C (2 min) at 60 °C/min.

2.6. Mass spectrometry

The LPS-derived oligosaccharides were analyzed using a linear ion-trap (LIT) mass spectrometer (LIT LXQ, Thermo Finnigan, San Jose, CA, USA) with electrospray (ESI) ionization and the conditions were as follows: electrospray voltage 5 kV; capillary temperature 275 °C; capillary voltage 1 V; and tube lens voltage 40 V; 100 μL of diluted sample; flow rate 8 μL/min. Nitrogen was used as nebulizing and drying gas. In tandem MS (MS^n) experiments, the collision energy used was set between 18 and 31 (arbitrary units). Data acquisitions were carried out on an Xcalibur data system. Prior to ESI-MS and MS^n analysis, dried oligosaccharides were dissolved in milli-Q water and de-salted on a Dowex cation-exchange resin (20−50 mesh, Bio-Rad, Hercules, CA, USA). The supernatants were recovered and 10 μL of each sample were diluted in water/methanol (1:1, v/v) containing 0.1 % formic acid. ESI-MS and ESI-MS^n spectra were acquired in the positive mode, scanning the mass range from *m/z* 100 to 1500 in ESI-MS experiments.

2.7. Carbohydrate microarray construction and analysis

To construct the carbohydrate microarray, here designated '*H. pylori* LPS microarray', the six structurally analyzed LPSs from *H. pylori* clinical isolates and 26695 strain were immobilized non-covalently onto 16-pad nitrocellulose coated glass slides, together with thirty-seven carbohydrate probes (Table S3). These included seven LPSs from other Gram-negative bacteria, twenty-three sequence-defined lipid-linked oligosaccharides, prepared as neoglycolipids (NGLs), and seven polysaccharides. Detailed information on the carbohydrate probes, microarray construction, printing conditions, imaging and data analysis, compliant with the Minimum Information Required for A Glycomics Experiment (MIRAGE) guidelines for reporting glycan microarray-based data (Liu et al., 2016), is available in Table S4.

Twenty-four carbohydrate-binding proteins, including plant and

human immune lectins, and monoclonal antibodies (mAbs), were analyzed in the *H. pylori* LPS microarray. Information on their names, sources, conditions of analysis and their reported carbohydrate recognition is detailed in Table S5. The microarray binding analyses were performed as described (Liu et al., 2012). The plant lectins and the anti-glucan 16.412E-IgA and 3.4.1G6-IgG3 antibodies were all biotinylated, thus a single-step overlay protocol was carried out. Briefly, for these, the microarray slides were blocked for 60 min with 1 % or 3 % (w/v) bovine serum albumin (BSA, Sigma) in 10 mM HEPES-Buffered Saline (pH 7.3), 150 mM NaCl, 5 mM $CaCl_2$ (referred to as HBS) with or without casein (Pierce), followed by the overlay, for 90 min, with the different proteins diluted in the blocking solutions to the final concentrations given in Table S5.

The human lectins and the antibodies were analyzed using biotin-streptavidin detection system. In brief, after the blocking step, the microarrays were overlaid for 90 min with the antibody solutions prepared to the final concentrations/dilutions given in Table S5, followed by incubation with the corresponding detection reagents in specified blocking solutions for 60 min. The human galectin-3 was analyzed at 50 μg/mL in 1 % (w/v) BSA, 0.02 % (v/v) casein in HBS, 5 mM, with detection using mouse monoclonal anti-poly-Histidine (Ab1), for 60 min, followed by biotinylated anti-mouse IgG (Ab2) for 60 min, both at 10 μg/mL in the blocking solution (Table S5).

For analysis of the 24 human sera from Hp + and Hp- individuals, the slides were blocked with 3 % (w/v) BSA in 1 % casein (blocking solution) for 60 min, followed by the overlay with the sera diluted 1:100 in the blocking solution for 90 min. In preliminary analyses, the serum from one Hp+ and one Hp- individuals was analyzed for IgM and IgG antibodies detected using biotinylated anti-human IgM or anti-human IgG (1:200 in the blocking solution), respectively. As binding was distinct with detection of IgG, but not of IgM antibodies (Fig. S2), only the IgG antibody profiles against *H. pylori* and other bacterial LPSs of both infected and non-infected control groups were evaluated.

The detection reagents in the absence of the proteins or human sera were also analyzed to detect any nonspecific binding. For all the microarray analyses, the Alexa Fluor-647-labeled streptavidin (1 μg/mL, Molecular Probes, S21374), diluted in the corresponding blocking solutions (Table S5), was used for readout, using a GenePix® 4300A fluorescence scanner (Molecular Devices, UK). The parameters for recording the fluorescence images were selected considering the signal to noise ratio, and saturation of the spot signals in the different experiments (Table S4). The analyses were performed at ambient temperature, except for anti-i P1A ELL antibody that was at 4 °C. Microarray data analysis was performed using dedicated software developed by Mark Stoll of the Glycosciences Laboratory, as described (Stoll & Feizi, 2009).

2.8. Ethics statement

The *H. pylori* clinical isolates were recovered from gastric biopsies of patients with gastric symptoms being investigated at Hospital de Santo António (Porto, Portugal). All procedures were approved by the Ethical committees of Centro Hospitalar do Porto (Portugal) and written informed consent was received from all participants.

3. Results and discussion

3.1. Fractionation and structural analysis of H. pylori LPSs

The LPSs of the five *H. pylori* clinical isolates and from the 26695 were recovered from the aqueous phase after hot phenol-water extractions and fractionated on a Sephacryl S-300. Based on the chromatographic profiles (Fig. S3), the average Mw of each LPS was calculated and estimated as 300 kDa for 26695, 340 kDa for CI-117 and 14255, 290 kDa for 14382 and 20 kDa for 2191 and CI-5, pointing to different LPS structural features in the strains. This interstrain variability was visualized by SDS-PAGE analysis and silver staining (Fig. S4). Greater microheterogeneity and similar banding profile was observed with the LPS from strains 26695, CI-117, 14255, and 14382, when compared with those from 2191 and CI-5 strains. Irrespective of this variability, the LPSs studied showed the ladderlike arrangement characteristic of smooth-LPS (Amano, Yokota, & Monteiro, 2012; Hiratsuka et al., 2005; Mills, Kurjanczyk, & Penner, 1992; Monteiro et al., 2000). The differential migration of major bands in the mid region of the gel, is consistent with different O-antigen chain lengths of the LPS (Fig. S4).

Methylation analysis of the different Sephacryl S-300 LPS fractions showed a complex mixture of partially methylated alditol acetates (Table 1, columns a, and Fig. S5). These were grouped and assigned to the different regions of the LPS (Fig. 1) (Li et al., 2017). All *H. pylori* LPS contained fucose (Fuc), glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc) and heptose (Hep) residues but with distinct relative molar ratios of glycosidic linkages. Terminal Fuc (t-Fuc, 2 %–16 %), 3-linked Gal (8 %–41 %), 4-linked (2 %–36 %) and 3,4-linked GlcNAc (2 %–10 %), characteristic of the O-chain region of a smooth-LPS, were present in all strains as major structural units (Table 1, columns a, and Fig. S5). These findings point to the occurrence of type 2 (poly)LacNAc moieties, (-3Galβ1-4GlcNAcβ1-)$_n$, also known as i-antigen (Feizi, 1981), and type 2 Le^x antigen, -3Galβ1-4(Fucα1-3)GlcNAcβ1-, or to its type 1 chain Le^a isoform, -3 Galβ1-3(Fucα1-4)GlcNAcβ1- (Kabat, 1982; Watkins, 1980). The 2-linked Gal, indicative of a type 2 Le^y sequence, Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-, or its type 1 Le^b isomer, Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1- (Kabat, 1982; Watkins, 1980), was not detected by —GC-MS analysis. The unexpected absence of Le^y, particularly in strain 26695, was likely due to the very limited amounts of the starting material used for methylation analysis and/or to the high microheterogeneity of the studied LPS. In addition to Le determinants and LacNAc moieties, the occurrence of 4,6-linked GlcNAc residues in the LPS from 14255 strain (Table 1, column a) points to a partial glucosylation and/or galactosylation at the O-6 position of GlcNAc residues of the (poly)LacNAc backbone in replacement of some α-L-Fuc side-chains at O-3, similarly to what was observed for strains UA861 (Monteiro, Rasko, Taylor, & Perry, 1998) and 471 (Aspinall, Mainkar, & Moran, 1999).

A distinctive feature of 14382 LPS was the high abundance of 3-linked Gal residues. Out of 41 %, only 6 % seems to link 1,4-GlcNAc residues to form type 2 LacNAc backbone in a relative molar proportion of 1:1 (Table 1, column a). The remaining 35 % of 3-linked Gal units point to the occurrence of a 1,3-D-galactan. This structure was so far identified in the LPS from a mutant of the *H. pylori* strain SS1, the SS1 HP0826::Kan (Chandan, Jeremy, Dixon, Altman, & Crabtree, 2013). Considering the recent redefinition of *H. pylori* LPS domains, these galactans are assigned to the O-antigen variable region of LPS (Li et al., 2017).

The presence of 6-linked Glc residues in LPS from strains 26695 and 2191 (Table 1, column a), points to the existence of an α1,6-glucan, resembling dextran polymers previously observed (Altman, Chandan, Li, & Vinogradov, 2011; Monteiro, 2001). This homopolymer has been postulated to cap, through a nonreducing DD-Hep residue, the Trio moiety DD-Hep-Fuc-GlcNAc, which in turn is attached to the 2,7-linked Hep of the short core OS of the LPS (Glc-Gal-D-D-Hep-LD-Hep-LD-Hep-Kdo) (Fig. 1) (Li et al., 2017). Also, the detection of 3-DD-linked Hep residues in these strains points to a heptoglycan linked to the nonreducing extended α1,6-glucan chain in the O-antigen (Table 1). This structural linearity of heptan-glucan-Trio region has been reported to the recently reinvestigated LPS structures from the western strains 26695 (Altman et al., 2011a; Li et al., 2017), SS1 (Altman et al., 2011b) and O:3 (Altman, Chandan, Li, & Vinogradov, 2013), as depicted in Fig. 1.

Notably, 2-linked ribofuranosyl units, 2-Rib*f*, were identified in the LPSs of all clinical isolates, except 2191, with only trace amount (Table 1, column a), suggesting the occurrence of β1,2-D-ribans (with 3–5 Rib*f* units), a homopolymer found in SS1 strain (Altman et al.,

Table 1
Glycosidic linkage profile (relative molar ratio, %) of Sephacryl S-300 and Bio-Gel P2 fractions of the studied *H. pylori* LPS.

LPS domain	Linkage type	Relative molar ratio (%)*							
		14255 (a)	14382 (a)	CI-117 (a)	2191 (a)	CI-5 (a)	CI-5 (b)	26695 (a)	26695 (b)
	Lewis antigens & (poly)LacNAc								
	t-Fuc	8.4	7.5	4.5	2.4	15.9	9.0	7.6	4.3
	t-Gal	2.4	1.6	0.6	1.9	6.7	3.9	1.2	1.9
	2-Gal	-	-	-	-	-	-	-	-
	3-Gal	16.3	41.0	18.9	13.8	25.2	15.0	8.0	10.2
	2,3-Gal	0.4	0.6	0.5	0.3	0.2	0.5	0.4	0.7
	3,6-Gal	0.2	-	-	-	0.3	0.3	-	-
	2-Glc	-	-	-	-	-	-	0.2	-
	t-GlcNAc	0.1	0.2	0.3	0.5	-	0.6	2.1	3.9
O-antigen	4-GlcNAc	9.5	6.1	29.9	35.8	7.9	17.6	2.0	9.4
	3,4-GlcNAc	9.9	3.1	5.5	2.1	10.2	22.8	10.4	11.4
	4,6-GlcNAc	8.8	-	0.6	0.4	-	0.3	-	-
	3,4,6-GlcNAc	1.9	-	-	-	-	-	-	-
	Riban								
	t-Rib*f*	0.3	0.7	0.4	-	0.6	0.2	-	-
	2-Rib*f*	8.5	13.4	9.7	tr	15.9	10.8	-	-
	Glucan								
	6-Glc	0.4	0.4	0.6	16.1	0.2	-	6.4	9.3
	Amylose-like glycan								
	4-Glc	1.3	0.5	0.5	2.4	0.5	0.6	1.0	1.0
	Mannose-rich glycans								
	t-Man	0.2	-	-	-	0.1	-	-	-
	Heptan								
	3-dd-Hep	-	-	-	3.8	-	-	0.8	1.3
	Trio								
	6-dd-Hep	-	-	0.8	5.7	-	-	1.8	1.4
	3-Fuc	1.0	1.8	1.1	0.6	1.5	1.1	0.4	0.7
	3-GlcNAc	1.5	0.7	2.6	3.4	0.6	1.5	-	-
	3-Glc	1.3	2.4	1.7	1.3	1.8	1.4	0.6	0.8
	2-dd-Hep	1.6	2.4	1.2	0.7	1.5	1.0	1.6	1.7
	t-Glc	8.4	2.4	3.1	2.3	0.6	0.3	9.4	7.7
	4-Gal	1.1	2.5	2.1	0.7	1.2	0.7	2.6	2.5
	7-dd-Hep	3.9	4.9	4.8	0.3	3.3	3.2	6.5	5.7
Core	2,7-dd-Hep	5.4	3.2	-	-	4.1	6.9	6.0	11.0
	2-ld-Hep	1.1	1.8	1.2	1.4	1.2	0.7	2.4	3.4
	3-ld-Hep	-	-	-	0.3	-	-	-	-
	t-dd-Hep	-	0.3	0.3	1.3	-	-	0.9	1.0
Lipid A	6-GlcNAc	4.9	1.4	8.1	-	-	-	25.5	8.0
	4-Xyl	0.2	-	0.1	0.7	-	-	0.6	-
Not	3,4-Glc	0.7	0.7	0.7	0.4	0.4	0.5	0.9	-
assigned	4,6-Glc	-	-	-	0.7	-	-	-	-
sugars	3,6-Glc	-	-	-	0.4	-	-	-	-
	2-Rib*p*	0.1	0.3	-	-	0.2	0.4	-	-

* The relative molar ratio of each linkage type was calculated by dividing the area of the corresponding chromatogram peak by the sugar residue molecular weight. (a), LPS fractions of Sephacryl S-300 after treatment with nucleases and proteinase K; (b), LPS fractions of Bio-Gel P2 after hydrolysis with 50 mM TFA, 65 °C, 1 h. The LPS domains were arranged according to the recently published redefinition of these (Li et al., 2017). Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; DD-Hep, D-*glycero*-D-*manno*-heptose; LD-Hep, L-*glycero*-D-*manno*-heptose; Man, mannose; Rib, ribose; Xyl, xylose; tr, traces; '- 'not detected. The series of the sugars was considered the same as that occurring in nature: Fuc, L-series; hexoses, Rib and Xyl, D-series. For heptoses (DD- and LD-Hep) the assignment was based on published structures (Monteiro, 2001) and on GC–MS analyses. All hexoses showed to be present in pyranose form, and Rib occurred mainly in the furanose form.

2011b; Chandan et al., 2013). This homopolymer is now assigned to the O-antigen domain of the LPS (Fig. 1) (Li et al., 2017). Consistent with previous findings (Altman et al., 2011a; Li et al., 2017; Monteiro et al., 2000), there is no evidence of ribans on the LPS of 26695.

3.2. Mass spectrometry analysis of H. pylori LPS-derived oligosaccharides

Aiming to obtain oligosaccharide structures that could corroborate and further elucidate the proposed sequences above and be representative of those existing in the clinical isolates, such as ribans, the LPSs from strains 26695 and CI-5 were subjected to partial acid hydrolysis under mild conditions. Due to the acid-lability of certain sugar residues (*e.g.* Fuc), methylation analysis showed some differences in the relative molar ratio in LPS-derived high Mw fraction, remaining after hydrolysis (#A, Fig. 2) when comparing with that observed to the non-hydrolyzed LPS (Table 1, column b). However, the glycosidic linkages previously identified were the same after the partial acid hydrolysis treatment.

Considering the known heterogeneity and complexity of the LPS structures and the lower ionization efficiency high MW oligosaccharides, the low Mw oligosaccharide fractions (#B-#E, Fig. 2) were analyzed by ESI-MS^n. The oligosaccharides identified in the different LPS-oligosaccharide fractions were detected as protonated ions or sodium adducts (Table 2 and Fig. 3).

The proposed glycan sequences assigned below were based on GC-MS and ESI-MS^n analyses and on previously knowledge on *H. pylori* LPS structures (2011b, Altman et al., 2011a; Ferreira, Domingues, Reis, Monteiro, & Coimbra, 2010; Li et al., 2017). The ion at mass-to-charge ratio (*m/z*) 1063, identified in both 26695 and CI-5 oligosaccharide fractions, was assigned to a double repeating unit of Le^x determinants, [Le^x-Le^x + Na]$^+$, as part of the O-antigen of the LPS (Fig. 3a). The

occurrence of Le^x, instead of Le^a determinants was supported by the presence of the fragment ion at *m/z* 388 (Fig. 3a), which was previously reported as a diagnostic fragment ion for type 2 Le^x structures found in the strain NCTC 11637. Under similar MS analysis conditions, the type 1 Le^a isomer had a diagnostic ion at *m/z* 372 (Ferreira, Domingues et al., 2010), not detected here. The molecular ions and corresponding assigned structures [GlcNAc-Rib$_4$ + Na]$^+$ (*m/z* 772), [Rib$_3$-Gal-Glc-Hep-Fuc + Na]$^+$ (*m/z* 1099) and [Rib$_2$-Gal-Glc-Hep-Fuc + Na]$^+$ (*m/z* 967) were identified only in CI-5 fractions (Table 2 and Fig. 3b,c). The detection of these fragment ions corroborated the presence of a riban structural domain in the CI-5 LPS, that is likely to bridge the variable region of the O-chain encompassing (poly)LacNAc and/or Le determinants and the conserved Trio (DD-Hep-Fuc-GlcNAc) through the Gal-Glc residues, as depicted in Fig. 1.

Collectively, the structural analysis of the different LPSs provided insights into their monosaccharide composition, glycosidic linkage patterns and contributed to the assignment of their sequences in the core and O-chain LPS domains. LPS structural differences were observed between clinical isolates and 26695, such as the occurrence of ribans in the former, but not in the latter; as well as within the clinical isolates, such as the evidence of a galactan domain in the LPS from 14382 and CI-117 strains, reinforcing the structural complexity and heterogeneity of these polysaccharides.

3.3. Construction of *H. pylori* LPS microarray and analysis with sequence-specific proteins

To explore the recognition of *H. pylori* LPSs by proteins, a carbohydrate microarray was constructed, featuring the 6 analyzed LPSs and a selection of oligosaccharides with defined sequences, such as polylactosamine Ii-active, Le and blood group H-related, which are known to occur on the *H. pylori* O-chains. As controls, the microarray included LPSs from other Gram-negative bacteria, Glc- and GlcNAc-based homo-oligomers, and Glc- and Man-containing polysaccharides, which are structures that have been reported in *H. pylori* LPS and other pathogens (Table S3).

The microarray was first analyzed with a total of 22 carbohydrate sequence-specific proteins (Table S5). These included monoclonal antibodies (mAbs) directed at Le and blood group H-related sequences (the anti-Le^x mAbs anti-BG7, anti-SSEA1 and anti-L5, anti-Le^y, anti-SLe^x, anti-Le^a, anti-Le^b, anti-LNT, anti-H-type 1 and anti-H-type 2), linear polyLacNAc sequences (anti-i P1A ELL1), and α1,6-glucose sequences (anti-dextran 16.412E-IgA and 3.4.1G6-IgG3). Also analyzed were plant lectins with various specificities: *Aleuria aurantia* lectin (AAL) and *Ulex europaeus agglutinin*-I (UEA-I) towards fucosylated sequences; *Ricinus communis*-I (RCA$_{120}$), *Datura stramonium* lectin (DSL), *Lycopersicon esculentum* lectin (LEL), Wheat germ agglutinin (WGA), *Solanum tuberosum* lectin (STL) and *Griffonia Simplicifolia* lectin-II (GSL-II) towards LacNAc or β1,4-linked *N*-acetyllactosamine (GlcNAc) oligosaccharide sequences; and Concanavalin A (ConA) towards α-mannose. Distinct binding profiles were observed to the sequence-defined carbohydrate probes with a good correlation with reported specificities of the probes, which served as validation and quality control of the analysis (see Appendix B for the analysis with the full microarray set). The binding patterns of these proteins with the *H. pylori* LPSs are highlighted in Fig. 4.

Table 2
Identified ions in the ESI-LIT-MS spectra of low Mw glycan-rich fractions of Bio-Gel P2 from *H. pylori* 26695 and CI-5 (50 mM, 65 °C, 1 h), and the proposed glycan sequences.

m/z	Strain	
	26695	CI-5
552	[Le^x + Na]$^+$ (#E)	
653	[Hex$_4$ − 2H$_2$O + Na]$^+$ (#E)	
772		[GlcNAc-Rib$_4$ + Na]$^+$ (#C)
789	[Hex$_2$-Hep-Hep(P) − H$_2$O + H]$^+$ (#C)	
802		[LacNAc-Rib$_3$ + Na]$^+$ (#C)
917	[Le^x-LacNAc + Na]$^+$ (#B, #D)	
933	[LacNAc-Hex-LacNAc + Na]$^+$ (#C)	
967		[Rib$_2$-Gal-Glc-Hep-Fuc + Na]$^+$ (#C)
1063	[Le^x-Le^x + Na]$^+$ (#B, #D and #E)	[Le^x-Le^x + Na]$^+$ (#B)
1099		[Rib$_3$-Gal-Glc-Hep-Fuc + Na]$^+$ (#C)
1231		[Rib$_4$-Gal-Glc-Hep-Fuc + Na]$^+$ (#B)

The proposed glycan sequences were based on the ESI-MS^n analyses (Fig. 3) and on previously published *H. pylori* LPS structures (Altman et al., 2011b; Ferreira, Domingues et al., 2010; Li et al., 2017; Monteiro, 2001). The fractions (in brackets) are depicted in Fig. 2. Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; Hex, hexose; LacNAc, *N*-acetyllactosamine; Le^x, Lewis^x antigen, Gal-(Fuc)-GlcNAc; Rib, ribose; P, phosphate.

3.3.1. Fucosylated blood group-related sequences

The binding of AAL to all the *H. pylori* LPS was indicative of the presence of α-fucose. But the differential recognition obtained with the anti-Le mAbs, UEA-I and the anti-blood groups H-type 1- and H-type 2 mAbs (Fig. 4 and Appendix B), pointed to distinct fucosylated sequences in the *H. pylori* LPS.

The anti-Le^x mAbs, anti-SSEA1 (Gooi et al., 1981), anti-L5 (Streit et al., 1996) and anti-BG7, which exhibited specific binding to the probe with the Le^x sequence (LNFPIII, #16 Appendix B), showed good binding to the LPSs from 14255, 2191 and 26695, in addition to weak, but detectable binding to the LPS from CI-5 (Fig. 4 and Appendix B). These indicated the presence of Le^x-related antigens in these LPSs, but not in those from 14382 and CI-117 clinical isolates, corroborating the proposed glycan sequences in the structural analysis. Although methylation analysis was elusive, detection of binding with anti-Le^y was readily demonstrated to all *H. pylori* LPSs, pointing to the occurrence of these antigens. Binding to LPSs was not detected with anti-Le^a nor anti-Le^b mAbs, suggesting the absence of type 1 Le$^{a/b}$ antigens, and that the Le

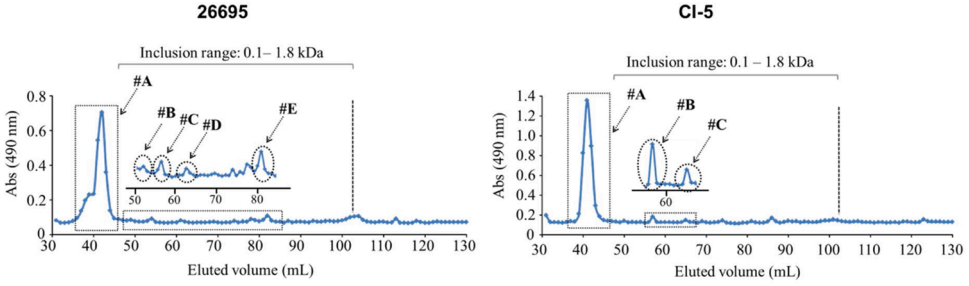
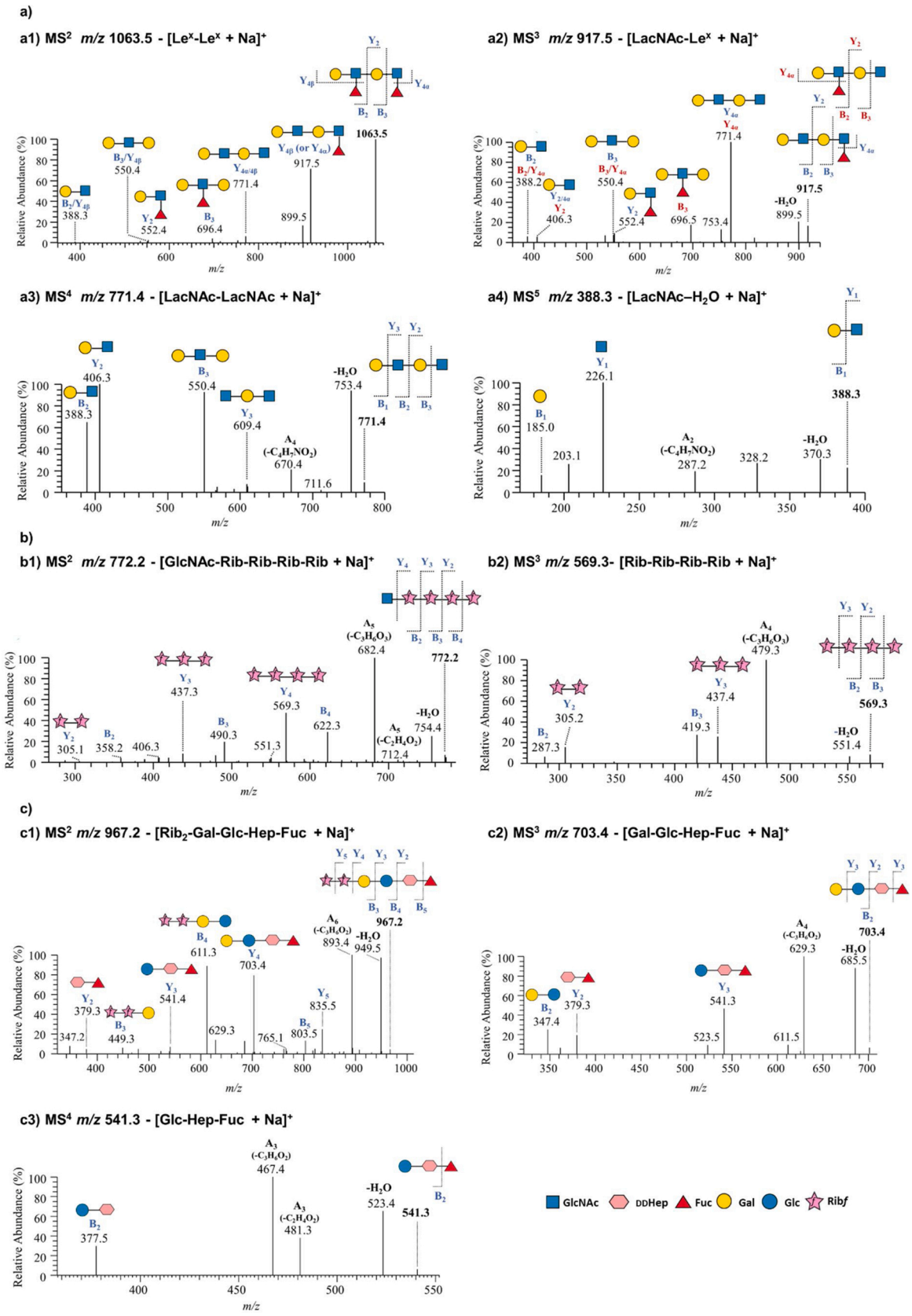


Fig. 2. Chromatographic profiles of *H. pylori* LPSs from 26695 and CI-5 strains obtained by gel filtration on Bio-Gel P2 after partial acid hydrolysis with 50 mM TFA for 1 h at 65 °C. Absorbance was measured at 490 nm to assess the content of total sugars. #A is the high Mw fraction analysed to their glycosidic linkages by GC–MS (Table 1); #B-#E are low Mw fractions analysed by ESI-MS^n (Table 2 and Fig. 3).



(caption on next page)

Fig. 3. Positive-ion mode ESI-LIT-MS^n spectra of representative *H. pylori* oligosaccharide fractions of Bio-Gel P2 (Fig. 2 and Table 2). The proposed assignement of the glycan moities were based on the ESI-MS^n analyses and on previously published *H. pylori* LPS structures (Altman et al., 2011b; Ferreira, Domingues et al., 2010; Li et al., 2017; Monteiro, 2001): **a)** di-Lewis^x antigen, **b)** -GlcNAc-Rib$_4$-, **c)** -Rib$_2$-Gal-Glc-Hep-Fuc-. **a1)** ESI-MS^2 spectrum of the ion at *m/z* 1063.5, assigned to [Le^x-Le^x + Na]$^+$; **a2)** ESI-MS^3 spectrum of the product ion at *m/z* 917.5, assigned to [Le^x-LacNAc + Na]$^+$, derived from the ion at *m/z* 1063.5; **a3)** ESI-MS^4 spectrum of the product ion at *m/z* 771.4, assigned to [LacNAc-LacNAc + Na]$^+$, from the product ion at *m/z* 917.5, derived from the ion at *m/z* 1063.5; **a4)** ESI-MS^5 spectrum of the product ion at *m/z* 388.3, assigned to [LacNAc−H$_2$O + Na]$^+$, from the product ion at *m/z* 771.4, derived from the product ion at *m/z* 917.5, originated from the ion at *m/z* 1063.5; **b1)** ESI-MS^2 spectrum of the ion at *m/z* 772.2, assigned to [GlcNAc-Rib$_4$ + Na]$^+$; **(b2)** ESI-MS^3 spectrum of the product ion at *m/z* 569.3, derived from the ion at *m/z* 772.2; **c1)** ESI-MS^2 spectrum of the ion at *m/z* 967.2, assigned to the structure [Rib$_2$-Gal-Glc-Hep-Fuc + Na]$^+$; **(c2)** ESI-MS^3 spectrum of the product ion at *m/z* 703.4, from the ion at *m/z* 967.2; **(c3)** ESI-MS^4 spectrum of the product ion at *m/z* 541.3 from the product ion at *m/z* 703.4 derived from the ion at *m/z* 967.2. Product ion nomenclature follows that proposed by Domon and Costello (Domon & Costello, 1988). The most common fragmentation pathways, Y- and B-type glycosidic cleavages and A-type cross-ring cleavages (da Costa et al., 2012) are shown. The representation of glycans follows the guidelines of Symbol Nomenclature for Glycans (Neelamegham et al., 2019). Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; DD-Hep, D-*glycero*-D-*manno*-heptose; Rib*f*, ribofuranose.

backbone-type structures are of type 2. In fact, the expression of Le$^{x/y}$ and the absence of Le$^{a/b}$ antigens were reported for strains 26695 (Li et al., 2017), 11637 (Aspinall, Monteiro, Pang, Walsh, & Moran, 1996), SS1 (Altman et al., 2011b) and J99 (Monteiro et al., 2000). The results are in accordance with the screening analyses using anti-Le antibodies, which have shown that type 2 Le$^{x/y}$ are the dominant phenotypes in western hosts, contrasting to Asian population that carry predominantly type 1 Le$^{a/b}$ antigens (Monteiro, 2001).

The UEA-I and anti-H-type 2 mAb, both of which recognize H-type 2 (Fucα1-2Galβ1-4GlcNAc-) sequence as in LNnFP-I (#14, Appendix B, and Table S5), showed strong binding to the LPSs from CI-117 and 14382 clinical isolates (Fig. 4 and Appendix B). These LPSs were also strongly bound by the anti-H-type 1 mAb, which recognizes Fucα1-2Galβ1-3GlcNAc- sequence as in LNFP-I (#13, Appendix B, and Table S5). Binding could also be detected with H-type 2 and H-type-1 mAbs to CI-5 and 2191 LPS. The binding with anti-H-type 2 antibody could also be due to the expression of Le^y determinants in these LPS, as this antibody bound, albeit weakly, to Le^y probe (LNnDFH-I, #20, Appendix B), probably due to the shared Fucα1-2Galβ1-4- epitope. The microarray binding data obtained with these antibodies showed the occurrence of H-type 1 and H-type 2 determinants in *H. pylori* LPS. Intriguingly the latter, to our knowledge, has not been described to date. The expression of H-type 2 sequences may be a result of the incomplete biosynthesis of Le^y epitopes identified in these LPSs. Noteworthy was the negligible or no binding of these blood-group H specific proteins to the LPSs from 14255 and 26695 which gave the strongest binding with anti-Le^x antibodies (Fig. 4 and Appendix B). None of the studied LPSs were bound by anti-sialyl Le^x mAb nor by anti-LNT mAb (Fig. 4 and Appendix B), pointing to the absence of sialylated Le^x and non-fucosylated type-1 structures (Galβ1-3GlcNAc), respectively.

The diverse fucosylation profile within *H. pylori* cell surface is regarded as a form of molecular mimicry to evade/modulate the host immune response, contributing to the pathogenesis and virulence of the bacterium (Moran, 2008).

3.3.2. GlcNAc, LacNAc and polyLacNAc sequences

The linear type 2 polyLacNAc sequences of LPSs were detected using the anti-i P1A ELL mAb (Fig. 4, Appendix B and Table S5). This mAb had a distinctive binding profile with CI-117 and 14382 LPS (Fig. 4 and Appendix B). The anti-i P1A ELL mAb recognizes substituted linear polyLacNAc sequences, whereas the lectins DSL, LEL, WGA and STL, which recognize terminal or internal (poly)LacNAc (Table S5), bound not only to the CI-117 and 14382 LPSs, but also to 14255 LPS, followed by those from strains 2191 and CI-5 (Fig. 4 and Appendix B), supporting the proposed structures inferred by the structural analysis. The weak interaction with 2191 and CI-5 LPSs was probably related to their low average Mw and the less efficient retention on nitrocellulose after the different incubations and several washes during microarray analyses. The nonreducing terminal β-galactose in the LPS was detected with RCA$_{120}$ lectin (Table S5), which showed strong binding to 14382 LPS (Fig. 4 and Appendix B), likely related with the expression of a 3-galactan in this LPS, as suggested by methylation analysis. Negligible or no binding was detected to any of *H. pylori* LPS with GSL-II that has specificity for terminal α-or β4-linked GlcNAc residues.

3.3.3. α-Glucose and α-mannose-related sequences

The 16.412E-IgA and 3.4.1G6-IgG3A antibodies bound to α1,6-glucan sequences (#29 and #40, Appendix B), as expected, but did not recognize the LPSs studied here (Table 1 and Appendix B). The lack of binding with anti-16.412E-IgA, known to be specific for nonreducing terminal α1,6-linked glucan sequences, was predicted to the *H. pylori* LPSs, as the α1,6-glucan reported (Altman et al., 2011a; Monteiro, 2001)

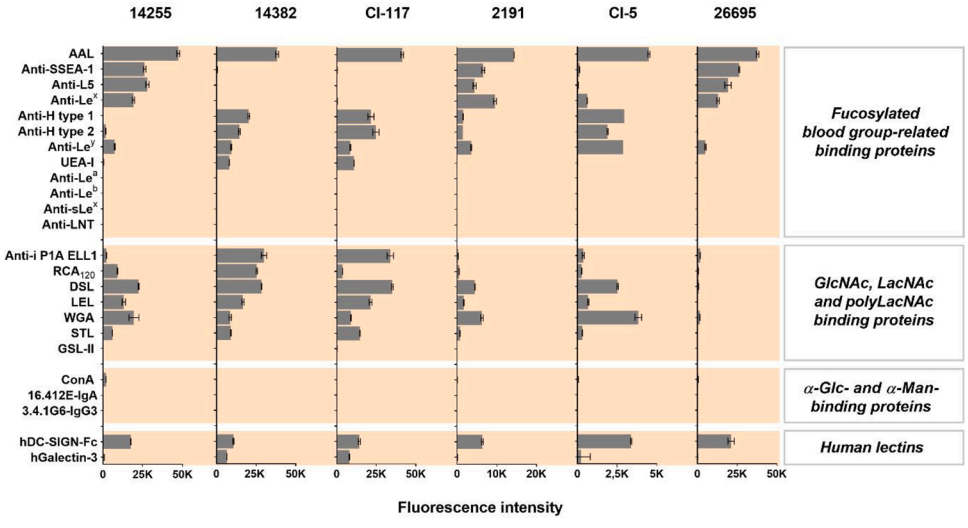


Fig. 4. Carbohydrate microarray analyses of the binding of glycan sequence-specific monoclonal antibodies, plant lectins and human immune lectins to LPS from *H. pylori* strains 14255, 14382, CI-117, 2191, CI-5 and 26695. The proteins are named on the left; apart from the two human immune lectins, dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) and galectin-3, the proteins are grouped according to their glycan recognition summarized on the right: fucosylated blood group-related sequences; GlcNAc, LacNAc and poly-LacNAc; and α-glucose and α-mannose sequences. The scales for the relative glycan binding intensities (fluorescence) are depicted at the bottom for each LPS; these are means of fluorescence intensities of duplicate spots of the high level of LPS arrayed (150 pg/spot). The error bars represent half of the difference between the two values. The full microarray set composed of 43 probes, and the binding data with all analyzed proteins is in Appendix B.

is internal, but the analysis with this antibody was included for quality control of the microarray. The anti-3.4.1G6-IgG3A preferentially binds internal α1,6-glucans with a chain length requirement of five or more residues (Palma et al., 2015). Although the expression of α1,6-glucan sequences in 26695 and 2191 LPSs was indicated by methylation analysis for 26695 and 2191 LPSs (Table 1), the negative array data with anti-3.4.1G6-IgG3A may be the result of a non-optimal presentation mode of these glycans in LPS for recognition by this antibody. ConA, which has a preference for terminal αMan-sequences as in probes #37-#39 (Appendix B), interacted very weakly with 14255 LPS only (Fig. 4).

Together, the microarray results with sequence-specific proteins provided additional and complementary information on *H. pylori* LPS structural domains. In addition to corroborating the occurrence of Le^x and (poly)LacNAc determinants in *H. pylori* LPS, microarrays revealed the expression of Le^y, as well as H-type 1 antigens and evidenced, for the first time, the presence of H-type 2 determinants mainly in LPSs from 14382 and CI-117 strains.

3.4. Recognition of H. pylori LPS by host innate immune receptors

To investigate the recognition of the studied LPSs by host proteins of the immune system, the *H. pylori* LPS microarray was interrogated with two human lectins: DC-SIGN and galectin-3. Among several biological functions, these lectins act as pattern recognition receptors of the immune system for pathogen-associated molecular patterns from bacteria, fungi and parasites with ability to trigger immune responses (van Kooyk & Geijtenbeek, 2003; Vasta, 2012).

The interaction between *H. pylori* and DC-SIGN has been suggested to be mediated by fucose-containing Lewis determinants on the O-antigen of LPS (Appelmelk et al., 2003). Consistent with its reported specificity (Geissner et al., 2019), the probes with Le^a (LNFP-II, #15), Le^b (LNDFH-I, #19), Le^x (LNFP-III, #16), and Le^y (LNnDFH-I, #20) sequences were strongly bound by DC-SIGN (Appendix B). Binding to H-type 2 (LNnFP-I, #14) and other Lewis-related oligosaccharides (#17 and #18) was also observed, in addition to α-Man- and α-Glc- containing sequences (#37-#39 and #24-#26, #41, respectively) and chitooligosaccharides (#22 and #23). All *H. pylori* LPSs were bound by DC-SIGN (Fig. 4 and Appendix B). The strongest binding was detected to 26695 and 14255 LPSs, likely associated with the high expression of Le^x. This was confirmed by sequence-specific proteins and pointed by methylation analysis (Table 1 and Fig. 3). Binding with DC-SIGN observed to CI-117 and 14382 LPSs are likely to be due to Le^y and H-type 2-containing sequences (Fig. 4 and Appendix B).

Galectin-3 is a β-galactoside-binding lectin suggested to interact with *H. pylori* through the O-antigen side chain of LPS (Fowler, Thomas, Atherton, Roberts, & High, 2006). In contrast to the broad binding of DC-SIGN to all six *H. pylori* LPSs, galectin-3 showed a restricted binding to those from strains CI-117 and 14382 (Fig. 4). Consistent with previous reports (Gimeno et al., 2019; Hirabayashi et al., 2002; Sato & Hughes, 1992; Stowell et al., 2008), galectin-3 bound to the non-fucosylated and fucosylated LacNAc-based sequence-defined NGL probes #8-#11, and #13, #14, respectively (Appendix B). Among these, the strongest binding was to H-type 1 (#13) and H-type 2 (#14)-related structures. Also, weak binding was detected to probe pLNFH-IV (#17, Appendix B), containing a Le^x linked to a LacNAc disaccharide (LacNAc-Le^x). Previous studies have shown weak binding of galectin-3 to Le^x determinants (Sato & Hughes, 1992), while others have reported no binding to LacNAc-Le^x-Le^x structures using glycan microarrays (Stowell et al., 2008). These findings reinforce the preference of galectin-3 for internal unsubstituted LacNAc units within polyLacNAc and that the recognition of this lectin is not influenced by terminal modifications, but, may instead, be affected by internal polyLacNAc alterations (Feizi et al., 1994; Gimeno et al., 2019). Considering the reported galectin-3 specificity and the microarray data above, the recognition of CI-117 and 14382 LPSs by galectin-3 could be due to the presence of internal LacNAc sequences in the O-chain region of the LPS, as pointed by strong binding with DSL and anti-i P1A ELL1, as well as to H-type 1 and H-type 2 sequences. Overall, CI-117 and 14382 LPSs showed similar patterns of glycan recognition with the proteins tested in the arrays (Fig. 4 and Appendix B). A striking difference seems to be related with terminal βGal residues, where binding with RCA_{120} was stronger to 14382 than CI-117 LPS (signal intensities: 25,366 and 3,711, respectively - Appendix B). This reinforces the expression of a 3-galactan in 14382 LPS suggested by methylation analysis (Table 1). Although no prior work has been done on interaction between galectin-3 and the 3-galactan recently proposed to occur in another *H. pylori* LPS (Chandan et al., 2013), the ability of this lectin to recognize polygalactosyl repeats (Galβ1-3) on lipophosphoglycans of the parasite *Leishmania major* (Pelletier & Sato, 2002), as well as the mammalian-like epitope Galα1-3 Gal on LPS O-antigen of the Gram-negative bacterium *Providencia alcalifaciens* (Stowell et al., 2014), supports this hypothesis.

3.5. Application of H. pylori LPS microarray for exploring adaptive immunity

Further to the interaction studies with host innate immune receptors, *H. pylori* LPS microarray was applied to investigate human serum IgG antibody responses to *H. pylori* LPSs, and to assess its utility for serological studies. A total of 24 serum samples were compared: 12 from *H. pylori*-infected (Hp+) individuals and 12 from non-infected (Hp-), grouped according to the clinicopathological parameters described in Table S2.

A distinctive serum IgG antibody response pattern was observed with Hp + cases to *H. pylori* LPS compared to other bacterial LPS (Fig. 5a and b and Appendix B). Remarkably, serum from Hp + cases elicited strong antibody reactivity against LPSs of all *H. pylori* clinical isolates with the means of IgG detection levels being significantly higher in the Hp+ group compared to those of Hp- controls (Fig. 5a). This correlates with the results using a commercial anti-*H. pylori* ELISA kit (Fig. 5a), that measures the IgG levels using as antigen a lysate of the *H. pylori* strain ATCC 43504. Although a higher IgG reactivity of sera from Hp+ individuals was also observed towards the reference strain 26695 LPS, compared to that of Hp- controls, the difference of the means was not statistically significant (Fig. 5a). A possible reason is that, in contrast to the clinical isolates, the *H. pylori* 26695 is a laboratory-adapted strain that has been subjected to multiple sub-cultivations on artificial media and may have thereby evolved to have an LPS with altered glycosylation. The 26695 LPS is indeed striking in its lack of binding by galectin-3 or weak binding to the LacNAc and polyLacNAc-binding proteins that are variously bound by the LPSs of the clinical isolates (Appendix B).

Comparing the IgG reactivity of Hp+ and Hp- groups against other bacterial LPSs, likely due to the cross reactivity with other LPS antigens, no significant differences were observed (Fig. 5b). These results show that the IgG antibody responses detected with Hp+ sera are specific for structures present in the LPS of *H. pylori* clinical isolates that are highly immunogenic in humans (Pece et al., 1997).

The occurrence of Lewis antigens in many *H. pylori* LPSs was previously suggested to elicit an autoimmune response in infected individuals (Appelmelk et al., 1996). However, other studies reported the absence of significant titers of antibodies to Lewis antigens in sera of *H. pylori*-infected individuals (Amano, Hayashi, Kubota, Fujii, & Yokota, 1997). Instead, two distinct antigens (termed highly and weakly antigenic epitopes) were observed in the O-chain region of all *H. pylori* smooth-form LPS, by reactivity with sera from humans with natural infection (Yokota et al., 2000). Furthermore, IgG and IgA antibodies observed against rough-LPS, besides smooth-LPS of the *H. pylori* NCTC 11637, indicated an immunogenic role of the core oligosaccharide (Pece et al., 1997) and reinforced previous findings that the antigenic epitopes in the LPS are unlikely to be immunologically associated with the Lewis antigen related structures (Amano et al., 1997; Yokota et al., 1998).

In this work, IgG antibody reactivities were not detected in serum

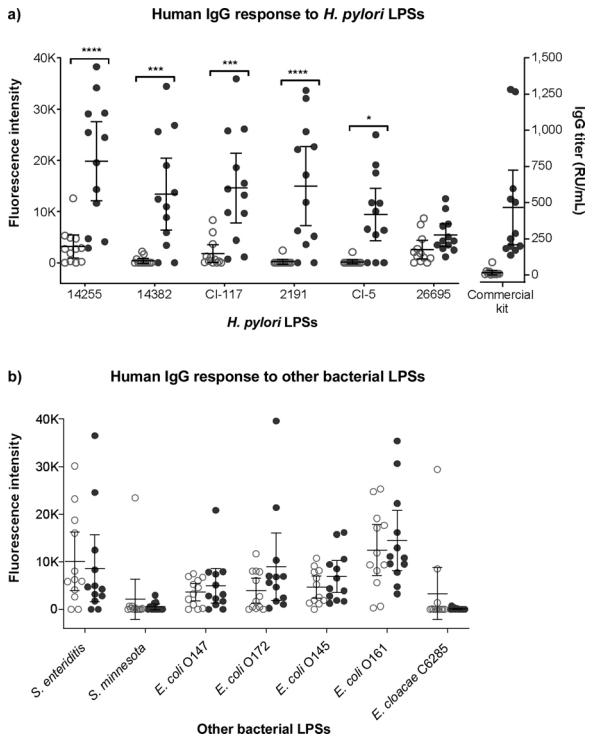


Fig. 5. Serum IgG antibodies detected to bacterial LPSs using the *H. pylori* LPS microarray. **(a)** LPSs from *H. pylori* strains; **(b)** LPSs from other Gram-negative bacteria. Sera of the *H. pylori*-infected (Hp+) cases are depicted as filled circles and those of non-infected (Hp-) controls, as open circles. The full microarray data are in Appendix B. For each case, the binding signal is the mean of fluorescence intensities from duplicate spots at the high level of LPS arrayed (150 pg/spot). The mean value of IgG reactivity for each serum group ± SD was calculated with 95 % confidence level. ****$p < 0.0001$, ***$p = 0.0001$ and *$p = 0.0136$, determined using two-way ANOVA followed by Sidak's multiple comparisons test. IgG titers, determined in relative units per mL (RU/mL) against a lysate of the *H. pylori* strain ATCC 43504 using a commercial anti-*H. pylori* ELISA kit, and that were used for case grouping in Table S2, are included in **(a)** for pattern comparison purposes.

from Hp+ individuals towards the fucosylated sequence-defined NGL probes (#13-#21, Appendix B), among them monomeric Le^x, SLe^x, Le^a, Le^b and Le^y, nor to other oligosaccharide sequences, such #11 and #12 (i-antigen), that were included in the microarray, and that may be found in the *H. pylori* LPS O-chain (Appendix B). These results are in agreement with a previous study of sera from *H. pylori*-infected patients with gastric pathologies (Pece et al., 1997): IgG antibodies against long polymeric, repeating Le^x antigen chains were detected, but not to short monomeric Le^x antigen chains. Long, repeating Le^x antigen chains were not included in this microarray.

The utility of carbohydrate microarrays for antibody profiling against bacterial glycans with potential for serodiagnosis of bacterial infections is increasing (Campanero-Rhodes, Palma, Menendez, & Solis, 2019). Our study clearly demonstrates the suitability of carbohydrate microarrays for serological studies, enabling detection of a specific anti-*H. pylori* LPS IgG response in sera from *H. pylori*-infected individuals. This microarray approach holds potential for rapid screening of multiple clinical samples to study host immune responses and to assess the seroprevalence of anti-LPS antibodies after the exposure to different *H. pylori* strains and to other bacteria. Furthermore, investigation of serum responses triggered by different isotypes of immunoglobulins against *H. pylori* LPSs would be valuable for associating clinical strains with different gastric diseases (Yokota et al., 1998).

4. Conclusion

The present study extends current knowledge on the chemical structures of *H. pylori* LPS by combining structural analysis and carbohydrate microarray analyses with carbohydrate sequence-specific proteins. All LPSs tested expressed $Lewis^{x/y}$ and *N*-acetyllactosamine determinants. Interestingly, ribans were detected in LPSs from all clinical isolates, as distinct from the 26695 LPS. There was evidence for the presence of 1,3-D-galactans and the blood group H-type 2 sequence in two of the clinical isolates, the latter not yet reported for *H. pylori* LPS. The carbohydrate microarray analyses showed differences of LPSs of the studied *H. pylori* strains in their recognition by the immune lectins DC-SIGN and galectin-3. Furthermore, the sera from *H. pylori*-infected patients were shown to contain IgG antibodies to *H. pylori* LPSs that were distinct from those directed at LPSs from other bacteria, demonstrating the potential of the microarray approach for serological studies. This work paves the way to applying carbohydrate microarrays combined with methods of structural analysis in *H. pylori* research while expanding their contents to include well characterized LPS-derived fragments of *H. pylori* LPS isolated from patients with more severe clinical outcomes. This will provide further analytical insights into the *H. pylori* LPS composition and help elucidate clinically relevant immunogenic components in these polysaccharides. The use of these complementary approaches will contribute to a better understanding of the molecular complexity of the LPSs and involvement in pathogenesis.

CRediT authorship contribution statement

Lisete M. Silva: Conceptualization, Data curation, Investigation, Formal analysis, Software, Validation, Project administration, Writing - original draft, Writing - review & editing. **Viviana G. Correia:** Data curation, Investigation, Software, Validation, Writing - review & editing. **Ana S.P. Moreira:** Investigation, Writing - review & editing. **Maria Rosário M. Domingues:** Writing - review & editing, Resources. **Rui M. Ferreira:** Resources, Writing - review & editing. **Céu Figueiredo:** Resources, Writing - review & editing. **Nuno F. Azevedo:** Resources, Writing - review & editing. **Ricardo Marcos-Pinto:** Resources, Writing - review & editing. **Fátima Carneiro:** Resources, Writing - review & editing. **Ana Magalhães:** Resources, Writing - review & editing. **Celso A. Reis:** Resources, Writing - review & editing. **Ten Feizi:** Conceptualization, Data curation, Writing - review & editing. **José A. Ferreira:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing. **Manuel A. Coimbra:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing. **Angelina S. Palma:** Data curation, Conceptualization, Investigation, Software, Validation, Supervision, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. and Appendix B Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.117350.

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