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Bioaccumulation of Amylose-Like Glycans by *Helicobacter pylori*

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Abstract

Background: *Helicobacter pylori* cell surface is composed of lipopolysaccharides (LPSs) yielding structures homologous to mammalian Lewis *O*-chains blood group antigens. These structures are key mediators in the definition of host-microbial interactions and known to change their expression pattern in response to environmental pressure.

Aims: The present work is focused on the identification of new *H. pylori* cell-surface glycosides. Special attention is further devoted to provide insights on the impact of *in vitro* subcultivation on *H. pylori* cell-surface phenotypes.

Methods: Cell-surface glycans from *H. pylori* NCTC 11637 and two clinical isolates were recovered from the aqueous phase resulting from phenol:water extraction of intact bacteria. They were evaluated in relation to their sugars and glycosidic-linkages composition by CG-MS, size-exclusion chromatography, NMR, and Mass Spectrometry. *H. pylori* glycan profile was also monitored during subcultivation *in vitro* in agar and F12 liquid medium.

Results: All three studied strains produce LPS expressing Lewis epitopes and express bioaccumulate amylose-like glycans. Bioaccumulation of amylose was found to be enhanced with the subcultivation of the bacterium on agar medium and accompanied by a decrease in the expression of LPS *O*-chains. In contrast, during exponential growth in F12 liquid medium, an opposite behavior is observed, that is, there is an increase in the overall amount of LPS and decrease in amylose content.

Conclusions: This work shows that under specific environmental conditions, *H. pylori* expresses a phase-variable cell-surface α-(1→4)-glucose moiety.

Helicobacter pylori is a widespread Gram-negative bacterium that infects the gastric mucosa of humans leading to the onset of several gastric disorders, such as gastritis, gastric ulcers, and cancers [1,2]. Moreover, persistent infection with *H. pylori* is strongly associated with the risk of developing gastric cancer [2,3]. Therefore, the microorganism is recognized as a category (definitive) human carcinogen [4].

H. pylori is regarded as a highly fastidious organism, typically requiring 3 days of growth on complex media containing blood or serum in a low-oxygen atmosphere at 35–37 °C [5–7]. The fastidious nature of *H. pylori* has hampered the initial isolation of the microorganism from the human stomach and continues to be one of the major drawbacks in research progresses since its discovery. In fact, studies reporting the growth of the microorganism in broth, particularly in chemically defined liquid medium, are relatively scarce. A possible and most likely explanation for the difficulties encountered in the cultivation of *H. pylori* in vitro may reside on the inability to mimic special conditions encountered by this microorganism gastric niche. Thus, it can be considered that during growth outside the human reservoir *H. pylori* is submitted to a different environmental pressure.

As a Gram-negative bacterium, *H. pylori* expresses at its cell surface lipopolysaccharides (LPSs) that comprise, when biosynthetically complete, three structural domains: a polysaccharide (PS) known as *O*-chain, an oligosaccharide termed core, and a fatty-acid-rich endotoxin moiety named lipid A (LPS: *O*-chain → Core→Lipid A∼cell). Commonly found *O*-chains yield fucosylated glycoside structures analogous to some mammalian histo-blood-group antigens, such as Lewis

determinants (Le^a, Le^b, Le^x, sialyl Le^x, Le^y) [8]. By mimicking the human cell-surface glycosylation in the stomach they act as molecular decoys for the immune system and therefore contribute to the persistence of the infection [9,10]. Another feature of some *H. pylori* strains is the presence of an additional chain of D-*glycero*-D-*manno*-heptose (DD-Hep), which has been reported to connect the *O*-chain to the LPS core [11]. Also, some LPS strains have been shown to express elongated α-glucan chains linked to the core [8,12,13]. LPS α-glucans can be either found *O*-6-linked [8,12] or, as recently observed for *H. pylori* serotype O:2, as an elongated *O-chain* composed of alternating *O*-2- and *O*-3-linked α-D-glucopyranose (α-D-Glc*p*) residues [→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→]$_n$ [13]. Proof have also been presented for the expression of a highly branched cell-surface α-mannan composed of terminal, *O*-2-, *O*-6-, and *O*-2,6-substituted mannose (Man) residues [14].

Cell-surface glycosylation is known to be involved in the control of various biologic and physiologic events such the modulation of protein conformational and functional properties [15,16], mediation of cell–cell adhesion and cell–host interaction [16,17], regulatory [16,18] and immunologic [16,19,20] phenomena. Alteration of the cell-surface glycosylation patterns often occurs as a result of the adaptive responses to environmental changes comprising events such as the expression of capsular polysaccharides as well as excretion of polysaccharides to the surrounding media as exopolysaccharides [21]. These events are thought to confer extra protection against harsh environments and external aggressions. Production of exopolysaccharides is strongly correlated with the formation of biofilms responsible for the colonization of unfriendly environments and the promotion of bacterial life on community [22].

In this work, we devote our attention to the identification and structural characterization of novel *H. pylori* cell-surface glycosides mainly produced as a result of environmental adaptation to growth in vitro solid and liquid media. Such insight can provide some enlightenment on the bacterial colonization strategies outside the human host, namely through the production of exopolysaccharides involved in the formation of biofilms or expression of capsular polysaccharides under very specific environmental conditions.

Materials and Methods

Bacterial Cultures

Reference strain *H. pylori* NCTC 11637 was obtained from the National Collection of Type Cultures (London, UK), clinical isolate 968 was provided by the Centre of Biological Engineering of the University of Minho (Braga, Portugal), and clinical isolate 14255 was provided by IPATIMUP (Porto, Portugal).

Bacterial Growth in CBA

H. pylori cells were maintained as stock cultures in trypticase soy broth with 20% glycerol, recovered on prewarmed Gelose Columbia solid media supplemented with 5% horse blood (bioMérieux, France) and incubated at 37 °C for 72 hours, on a microaerophilic atmosphere generated by a CampyGen gas pack (Oxoid, Basingstoke, UK). Cells were subcultivated after 48-hour incubation periods in the conditions described before. The resulting biomass was harvested to sterile and filtered (0.22 μm) distilled water.

Bacterial Growth in F12 Liquid Media

H. pylori reference strain NCTC 11637 and clinical isolate 968 were grown on Ham's F12 defined liquid media without the addition of enhancing growth factors. The medium was inoculated with cellular extracts obtained from Columbia blood agar (CBA) growth media. Cells were allowed to grow for 24 hours in a glass flask under microaerophilic atmosphere by gentile stirring at 37 °C as described by Testerman et al. [6].

Purity Assessment of Bacterial Growth

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 Guimaraes et al. [23]. For every experiment, a negative control was performed simultaneously where all the steps described before were carried out but where no probe was added during the hybridization procedure. Microscopy visualization was performed using an Olympus BX51 (OLYMPUS Portugal SA, Porto, Portugal) epifluorescence microscope equipped with one filter sensitive to the Alexa fluor 594 signaling molecule attached to the PNA probe (excitation 530–550 nm; barrier 570 nm; emission LP 591 nm).

Extraction and Purification of Cell-Surface Glycans

Cell-surface glycans were isolated from cells using the hot phenol : water extraction according to Westphal and Jann [24]. 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 Laboratories, Hercules, CA, USA) (0.6 m in length and 1.0 cm in diameter) column using distilled water as the eluting solvent at a constant flow rate of 0.33 mL/minute. The initial material (~2.5 mg) was suspended in 400 μL of degassed distilled water and introduced in the column. Fractions of 2.8 mL were collected. The mass content in the fractions was estimated on evaporative light scattering detector SEDEX55 (SEDERE, Alfortville, France) and further assayed for total sugars with the phenol–H_2SO_4 method [25]. Exclusion and total volume were calibrated with blue dextran and glucose, respectively.

Sugars Composition and Linkage Analysis

Sugars composition analysis was performed by the alditol acetate method as described by Harris et al. [26]. The hydrolysis was done in 4 mol/L of trifluoroacetic acid (TFA) at 100 °C for 3 hours, followed by reduction with $NaBH_4$ and subsequent acetylation with acetic anhydride in the presence of 1-methylimidazole. Alditol acetates derivatives were analyzed by gas chromatography-mass spectrometry (GC-MS), performed in an Agilent Technologies (Santa Clara, CA, USA) 6890N Network Gas Chromatograph connected to an Agilent 5973 Selective Mass Detector. The GC was equipped with a DB-1 capillary column (30 m in length, 0.32 mm in internal diameter, and 0.25 μm film thicknesses). The samples were injected in splitless mode (splitless time is 1.00 minute), with the injector and detector operating at 220 and 230 °C, respectively, using the following temperature program: 100 °C (1 minute) → 150 °C at 5 °C/minute (8 minutes) → 159 °C at 0.5 °C/minute (5 minutes) → 250 °C at 5 °C/minute (2 minutes) → 320 °C at 2 °C/minute (2 minutes). The carrier gas (He) had a flow of 1.7 mL/minute, with an average linear velocity of 47 cm/second, and a solvent delay of 9 minutes. The column head pressure was 5.34 psi. The mass spectrometer was operated in the electron impact mode at 70 eV, scanning the mass range *m/z* 40–500 and performing 3.18 scans per second.

Linkage analysis was carried out by methylation with $NaOH/Me_2SO/CH_3I$, as described by Ref. [27]. The methylated polysaccharides were hydrolyzed with 2 mol/L of TFA at 121 °C for 1 hour, reduced by $NaBD_4$, and acetylated with acetic anhydride in the presence of 1-methylimidazole. The partially methylated alditol acetates were analyzed by GC-MS using the chromatographic conditions previously described, using the following temperature program: 45 °C (5 minutes) → 140 °C at 10 °C/minute (5 minutes) → 170 °C at 0.5 °C/minute (1 minute) → 280 °C at 15 °C/minute (5 minutes).

Iodine Test

A thin layer of fresh bacterial cells grown on solid medium was fixed on a cleaned sterile glass slide. Cells were then covered with Gram's iodine solution (0.33% (w/v) I_2 in 0.66% (w/v) KI aqueous solution; Sigma-Aldrich Química S.A., Sintra, Portugal) and incubated for 1 minute at room temperature. After incubation, the cells were washed with water to remove the excess of staining agent and observed by light microscopy.

Enzymatic Assays

Digestion with α-amylase from human saliva (Type IX-A, lyophilized powder, 1000–3000 units/mg protein; Sigma Aldrich) was performed in 50 mmol/L phosphate buffer at pH 6.8 for 3 hours at 20 °C. The enzyme was denatured by heating the solution for 15 minutes at 100 °C and the precipitate material was removed by centrifugation.

The enzyme-treated material was separated on a polyacrilamide Bio-Gel P-4 column (0.6 m in length and 1.0 cm in diameter) using water as an eluent in the conditions previously mentioned for the separation on the Bio-Gel P6 column.

NMR Spectroscopy

^1H spectra were recorded on a Bruker (Bruker BioSpin GmbH, Rheinstetten, Germany) AMX 400 spectrometer at 295 K using standard Bruker software. Prior to performing the nuclear magnetic resonance (NMR) experiments, the samples were lyophilized thrice with D_2O (99.9%). The HDO peak was used as the internal reference at δ_H of 4.76 ppm.

Mass Spectrometry

MS assays were performed in a linear ion trap (LIT) electrospray (ESI) spectrometer in positive mode. Samples were dissolved in 1 : 200 of water : methanol and introduced into the MS at 10 μL/min. Typical ESI conditions were as follows: nitrogen sheath gas 30 psi, spray voltage 5.5 kV, heated capillary temperature 350 °C, capillary voltage 1 V, and tube lens voltage 40 V. Collision-Induced Dissociation-Mass Spectrometry (CID-MS/MS) experiments were performed on mass-selected precursor ions using standard isolation and excitation procedures (activation *q* value of 0.25, activation time of 30 ms). The spectra were collected in the positive mode and collision energy used was 32 (arbitrary units). Data acquisition was carried out with Xcalibur data system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Statistics

Statistical significance was determined using paired Student's *t*-tests ($p = .05$). Between- and within-sample variances were determined by one-way ANOVA and comparison between standard deviations performed using an *F*-test ($p = .05$). Correlation between samples was determined by linear regression method.

Results

Carbohydrate Profile of Glycan-Rich Extracts from *Helicobacter pylori* Cells Grown in Solid Media

In this study, *H. pylori* reference strain NCTC 11637 and clinical isolates 968 and 14255 were evaluated in relation to their cell-surface glycan profile. The first step in the identification and structural characterization of cell-surface polysaccharides comprised the recovery of glycan-rich aqueous phase resulting from hot phenol : water extraction performed on intact bacterial cells. The overall sugars composition of such extracts is presented in Table 1. The different strains present distinct sugars composition. NCTC 11637 contains a large amount of Rib, possibly a contamination from RNA, glucose (Glc), and galactose (Gal) whereas 968 presents also Gal and Glc but is richer in *N*-acetylglucosamine (GlcNAc) and D-*glycero*-D-*manno*-heptose (DD-Hep) and 14255 is richer in Gal and GlcNAc. Lower amounts of fucose (Fuc), arabinose (Ara), Man, and L-*glycero*-D-*manno*-heptose (LD-Hep) have also been observed in all strains. The occurrence of Ara has never been reported to occur in *H. pylori*. The different sugar contents might reflect both strain-to-strain variability and biosynthetic microheterogeneity.

To highlight the structural features of these polymers, the linkage profile held by the identified sugars was further evaluated by GC-MS of the permethylated alditol acetate derivatives released after hydrolysis of the original glycans.

Linkage analysis has revealed, in all CBA grown strains, a prevalence of *O*-3-linked Gal and *O*-4-linked GlcNAc residues in proportions of approximately 1 : 1. Based on previous knowledge on *H. pylori* cell-surface glycans [8], these structures belong to *N*-acetyllactosamine (LacNAc) moieties [→3)Gal(1→4)GlcNAc(1→] characteristic of LPS *O*-chains. The identification of terminal Fuc residues and *O*-3,4-linked GlcNAc residues in proportions of approximately 1 : 1 further suggests the expression of Type 1 Le^a or Type 2 Le^x blood group determinants. The existence of residual amounts (<1%) of *O*-2,3-linked Gal indicates the expression of small amounts of Type 1 Le^b or Type 2 Le^y blood group determinants. This linkage composition is in accordance with what has been described for reference strain NCTC 11637 that is known to have *O*-chains containing LacNAc and Type 2 Le^x and Le^y [28]. The clinical isolate 968 presents a high percentage of *O*-7-linked DD-*manno*-Hep, suggesting the expression of an elongated heptoglycan usually found linking the core to the *O*-chains [8]. Low amounts (<5%) of other *O*-chain and core-related residues have also been identified and assigned as shown in Table 2.

Man was observed in all extracts in percentages that span from 5 to 11% of total sugars (Table 1). These residues are essentially found as terminal, *O*-2-, *O*-6-, and *O*-2,6-linked (Table 2). Monteiro et al. [14] have shown the occurrence of a mannan containing these characteristic linkages when studying a *H. pylori* mutant strain. Methylation analysis shows that the Ara residues occur mainly as *O*-2-substituted even though trace amounts of terminal and *O*-5-linked residues could also be observed.

In addition to LPS sugars, Man and Ara, high amounts of *O*-4-linked Glc residues [→4)-Glc*p*-(1→] were also common to all extracts (Table 2). The total amount of

Table 1 Relative sugar composition of glycan-rich water extracts of the *Helicobacter pylori* studied strains

	Molar ratio (%)								
H. pylori strains	Rib	Fuc	Ara	Man	Glc	Gal	GlcNAc	DD-Hep	LD-Hep
Growth in CBA									
NCTC 11637	39	5	1	11	19	18	4	2	1
968	2	3	1	7	13	20	24	20	10
14 255	4	9	2	5	7	31	32	7	3
Growth in F12 broth									
NCTC 11637	5	5	2	22	25	23	9	6	3
968	5	2	1	27	33	15	14	2	1

Rib, ribose; Fuc, fucose; Ara, arabinose; Man, mannose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; DD-Hep, D-*glycero*-D-*manno*-heptose; LD-Hep, L-*glycero*-D-*manno*-heptose; CBA, Columbia blood agar.

Table 2 Sugar linkage profile of the sugars in glycan-rich extracts from the *Helicobacter pylori* studied strains

	Relative molar ratio (%)				
	Growth in CBA			Growth in F12 broth	
Linkage type	NCTC 11637	968	14255	NCTC 11637	968
LPS *O*-chain					
T-Fuc	5	4	8	4	7
→3)Fuc	–	tr	1	–	3
→6)Glc	1	–	tr	5	4
T-Gal	2	1	2	1	1
→3)Gal	18	15	20	9	12
→2,3)Gal	1	1	tr	tr	1
→2,4)Gal	–	tr	tr	–	–
→3,4)Gal	–	tr	tr	–	–
→3,6)Gal	–	1	tr	–	–
T-GlcNAc	tr	1	–	2	–
→4) GlcNAc	10	11	16	9	5
→3) GlcNAc	tr	1	1	tr	1
→3,4)GlcNAc	5	5	14	2	8
→3,6)GlcNAc	–	tr	–	–	–
→4,6)GlcNAc	–	tr	–	–	–
LPS Core					
*T-Glc	6	3	2	3	2
→3)Glc	tr	v	2	tr	tr
→4)Gal	4	3	2	2	tr
T-D*D*-*manno*-Hep	–	–	–	1	tr
T-L*D*-*manno*-Hep	–	–	–	tr	–
→2)D*D*-*manno*-Hep	tr	5	3	4	1
→2)L*D*-*manno*-Hep	2	3	3	tr	1
→3)L*D*-*manno*-Hep	1	5	tr	tr	tr
→7)D*D*-*manno*-Hep	1	14	5	3	1
→7)L*D*-*manno*-Hep	–	7	tr	tr	1
→2,7)D*D*-*manno*-Hep	1	1	1	4	2
LPS Lipid A					
→6) GlcNAc	tr	3	4	4	1
Man-rich glycans					
T-Man	3	1	1	6	9
→2)Man	3	4	2	7	11
→6)Man	5	tr	1	4	5
→2,6)Man	4	1	tr	5	8
Amylose-like glycans					
→4)Glc	26	9	5	13	7
→4,6)Glc	1	tr	tr	–	–
Unassigned sugars					
T-Ara	tr	–	tr	tr	1
→2)Ara	1	1	6	1	3
→5)Ara	tr	tr	1	–	1
→2,3,5)Ara	–	–	–	–	–
→6)Gal	1	tr	tr	11	4

*Terminal Glc residues can also be addressed to the (1→4)-glucan or the (1→6)-dextran antenna in the lipopolysaccharides (LPS).
tr, vestigial amounts (> 0.5%); CBA, Columbia blood agar.

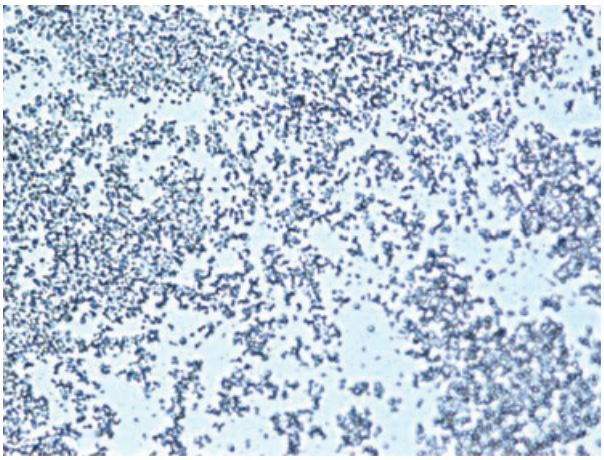

Figure 1 *Helicobacter pylori* cells stained with iodine. Characteristic blue color is indicative of the presence of α-(1→4)-D-Glc polysaccharides.

O-4-substituted Glc was found to vary from 5 to 26% of total sugars and its presence in glycan-rich water extracts is now being described for the first time for *H. pylori*. To confirm the presence of amylose-like glycans, *H. pylori* cells were treated with Lugol iodine solution. The blue-stained cells shown in Fig. 1 demonstrate the presence of amylose, with (1→4)-D-Glc in α configuration. Trace amounts of *O*-4,6-linked Glc residues were also identified in all samples, suggesting the existence of residual branching points in the glucan chain. Because the CBA culture medium contains starch, it is not possible to identify the origin of this material, if produced by the bacterium or uptaken from the medium.

Carbohydrate Profile of Glycan-Rich Extracts from *Helicobacter pylori* Cells Grown on Liquid Media

To determine if *H. pylori* was capable of biosynthesizing amylose-like glycans, the reference strain NCTC 11637 and the clinical isolate 968 were grown in Ham's F12 defined liquid media, which is devoid of polysaccharides in its composition. Cellular growth was promoted in the absence of external growth enhancers such as FBS, BSA, or cyclodextrin further fulfilling the requirement for a glycan-depleted environment. As it can be observed from sugars (Table 1) and linkage (Table 2) analysis, growth in liquid medium resulted, in both strains, in an increase percentage of Man and sugars belonging to the LPS moiety (Glc, Gal, GlcNAc, and Hep). The two strains also retrieved positive for the presence of amylose in liquid medium thus demonstrating the capability of those strains to synthesize amylose-like glycans in a polysaccharide-depleted medium.

To fully access the structural organization of the *O*-4-linked glucan, the water extracts from *H. pylori* reference strain NCTC 11637 was fractionated by size-exclusion chromatography on a Bio-Gel P6 medium.

Purification and Characterization of *Helicobacter pylori* Amylose-Like Glycans

Fractionation of the glycan-rich material in a Bio-Gel P6 resulted in the elution profile presented in Fig. 2, from which six fractions were identified and labeled from A to F.

The high-molecular weight fractions (A–C) were composed mainly of Gal (31–36%), Man (24–30%), Glc (17–25%), and also contained Rib (~7%). Lower amounts of GlcNAc (2–5%), Fuc (~3%), DD- and LD-Hep (1–3%), and Ara (~2%) have also been observed (Table 3). Linkage analysis allowed assigning the Fuc, Gal, GlcNAc, DD- and LD-Hep to the LPS moiety (Table 4) [8]. The Ara residues in fractions A–C were almost exclusively found *O*-2,3,5-linked (Table 4), a very uncommon substitution pattern in nature, although reported to occur in some seeds [29]. Residual amounts of terminal, *O*-2-, and *O*-5-linked Ara residues were also identified. Their presence throughout all fractions points towards that variously linked Ara residues are structural motifs of *H. pylori* strains.

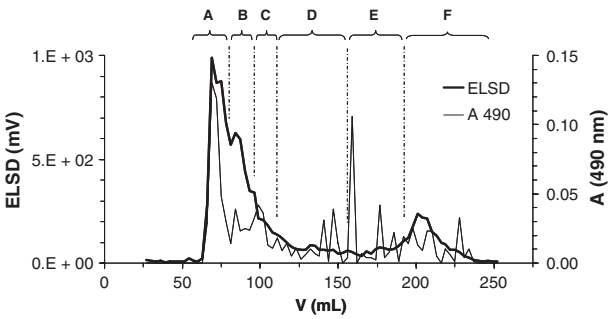

Figure 2 *Helicobacter pylori* NCTC 11637 glycan-rich extract elution profile on a Bio-Gel P6 column. Letters A–F correspond to isolated fractions.

The percentage of *O*-4-linked Glc residues increased from fractions A to F (Table 4). Glc was found to be the second major component of fractions A–C, and predominantly identified as being *O*-4-linked (~66–79% of the total Glc) The remaining Glc was either found in the *O*-3-linked form (7–14% of total Glc), assigned to the LPS, or *O*-6-linked form, assigned to dextran antenna in the LPS [12]. Terminal Glc was also present in percentages that span from 5% (fraction C) to 11% (fraction A) of total Glc and can either be found in the terminal ends of LPS core and dextran antenna as well as in the *O*-4-substituted glucan. Such structural diversity makes impossible the determination of the exact percentage of terminal Glc belonging to the glucan chain thus hindering the estimation of the molecular weight based on *O*-4-linked Glc/terminal Glc ratio. Linkage analysis on fractions A–C also showed residual amounts of *O*-4,6-disubstituted Glc (~2%) thus reinforcing that, even though almost exclusively linear, the glucan might exhibit vestigial branching. In fraction D, the *O*-4-linked Glc was the most abundant linkage. Still, it contains significant amounts of Man and Gal (16 and 19% of total sugars, respectively) and, in lower percentages, Rib (9%), Fuc (5%), Ara (4%), GlcNAc (2%), DD-Hep (3%), and LD-Hep (4%). Fractions E and F were composed almost exclusively of *O*-4-linked Glc (83 and 78%, respectively). Assuming a linear structure, the (1→4)/terminally linked Glc ratio allows inferring that the average length of these polymers can vary from 42 (7 kDa, fraction E) up to 28 residues (4 kDa, fraction F) Glc residues.

The polyacrylamide Bio-Gel P6 is expected to separate molecules based on their molecular weight, from 6 kDa (void volume) to 1 kDa (inclusion volume). These conditions are valid when no interactions occur between the stationary phase and the eluted sample. In

Table 3 Sugar composition of *Helicobacter pylori* NCTC 11637 glycan-rich extract fractions collected from a Bio-Gel P6 column (A–F)

	Molar ratio (%)								
Fraction	Rib	Fuc	Ara	Man	Glc	Gal	GlcNAc	DD-Hep	LD-Hep
A	8	3	2	30	17	36	2	1	1
B	7	4	1	30	19	31	5	2	1
C	6	3	2	24	25	32	4	1	3
D	9	5	4	16	38	19	2	3	4
E	3	6	6	2	78	2	–	1	2
F	59	1	4	1	34	1	–	–	–

Rib, ribose; Fuc, fucose; Ara, arabinose; Man, mannose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; DD-Hep, D-*glycero*-D-*manno*-heptose; LD-Hep, L-*glycero*-D-*manno*-heptose.

Table 4 Linkage composition of *Helicobacter pylori* NCTC 11637 glycan-rich extract fractions collected from a Bio-Gel P6 column (A–F)

	Relative molar ratio (%)					
Linkage type	A	B	C	D	E	F
LPS *O*-chain						
T-Fuc	2	9	3	2	–	–
→6)Glc	1	2	1	tr	–	–
T-Gal	6	6	4	8	–	–
→3)Gal	20	10	15	9	–	–
→4)GlcNAc	2	9	3	1	–	–
→3)GlcNAc	–	2	tr	2	–	–
→3,4)GlcNAc	2	5	2	–	–	–
→3,6)GlcNAc	–	–	–	–	–	–
→4,6)GlcNAc	–	1	–	–	–	–
LPS Core						
*T-Glc	2	2	2	4	–	–
→3)Glc	2	5	5	4	–	–
→4)Gal	15	6	3	8	–	–
→2)LD-*manno*-Hep	tr	2	tr	2	–	–
→7)DD-*manno*-Hep	tr	1	1	2	–	–
→3)LD-*manno*-Hep	0	tr	0	0	0	0
→2,7)DD-*manno*-Hep	tr	2	1	2	–	–
LPS Lipid A						
→6)GlcNAc(1→	tr	1	2	1	–	–
Man-rich glycans						
T-Man	7	5	6	5	–	–
→2)Man	10	15	11	3	–	–
→6)Man	8	2	3	5	–	–
→2,6)Man	8	5	6	2	–	–
Amylose-like glycans						
T-Glc	–	–	–	–	2	3
→4)Glc	12	9	26	36	83	78
→4,6)Glc	tr	tr	tr	–	–	–
Unassigned sugars						
T-Ara	–	–	3	–	–	–
→2)Ara	tr	tr	1	1	–	–
→5)Ara	–	tr	1	tr	7	9
→2,3,5)Ara	3	1	1	3	8	10

*Terminal Glc residues can also be addressed to the (1→4)-glucan or the (1→6)-dextran antenna in the lipopolysaccharides (LPS). tr, vestigial amounts (>0.5%); CBA, Columbia blood agar.

the present work, the eluent was water, which is known to not prevent molecular interactions. In that case, the sugar composition and linkage profile of the isolated fractions suggest that polyacrylamide Bio-Gel P6 separated the molecules based both on the molecular weight and their interactions with the gel medium. Linear molecules such as the described (1→4)-glucan tend to be retained more in the column (fractions D–F) whereas the more branched and voluminous LPS carrying a lipid A moiety are found in earlier eluted fractions (A–C). In agreement with these observations, highly branched Man appear preferentially in fraction A (~15% of *O*-2,6-disubstituted Man) whereas fraction B has only 2% of branched Man residues and no branching is found in fraction C.

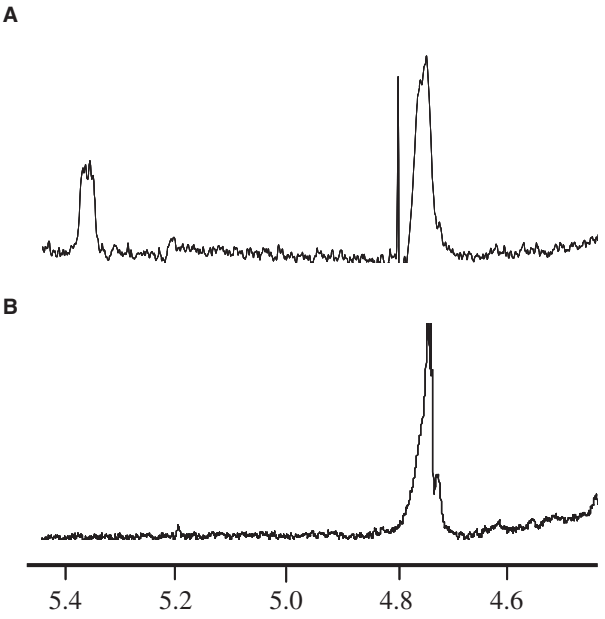

Figure 3 ^{1}H nuclear magnetic resonance anomeric region of fraction E (*A*) before and (*B*) after digestion with α-amylase.

To confirm the anomeric configuration and other structural features of the *H. pylori* glucan, fraction E, which exhibits a higher percentage of Glc (~79%) and contains only residual amounts of other sugars (>7%) was further used for NMR and ESI-MS analyses. The ^{1}H NMR spectra of fraction E retrieved a single anomeric resonance at 5.36 ppm thus demonstrating that Glc residues were in α configuration (Fig. 3A). Complementary ESI-MS and MS^2 experiments recorded in positive mode confirmed the presence of Glc sequences, as it can be seen by the peaks at *m/z* 851, 1013, 1175, 1337, and 1499 assigned to $[Glc_n+Na]^+_{5-9}$ ions (Fig. 4A). Even though the estimated chain length calculated from linkage analysis data was of 28 residues, some dispersion in the degree of polymerization is always expected, thus explaining the observation of molecules of lower molecular weight in the ESI-MS spectrum. Although the sugar composition of this fraction showed both Rib (8%) and Ara (6%) no evidences of a linkage between the Glc residues and a pentose was found.

To observe the extent of the contiguous *O*-4-linked Glc chains, fraction E was digested with an (1→4)-α-D-glucan glucanohydrolase (α-amylase). This enzyme is responsible for catalyzing the *endo*-hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. The digested material was then purified on a Bio-Gel P4 resulting in an elution profile composed of two

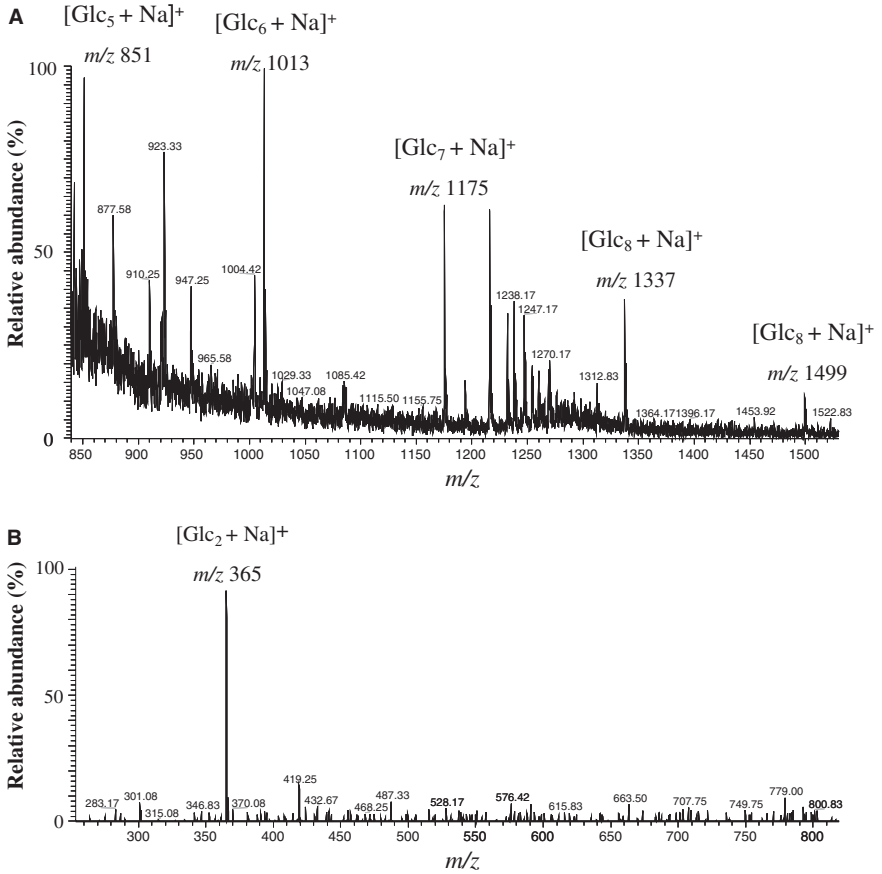

Figure 4 Electrospray-mass spectrometry spectra of (*A*) fraction E and (*B*) material recovered after digestion with α-amylase.

fractions, one corresponding to the void volume and another to nonincluded material (data not shown). The ^{1}H NMR spectrum of the material recovered from the void volume showed the disappearance of the α-anomeric signal, demonstrating the effectiveness of the enzymatic treatment (Fig. 3B). The nonretained material was composed exclusively of disaccharides resulting from the enzymatic hydrolysis of the oligomeric glucan as demonstrated by the ion at *m/z* 365 observed in the ESI-MS spectrum (Fig. 4B).

Bioaccumulation of Amylose under Subcultivation in Solid Medium

After having shown the expression of an amylose-like polysaccharide by *H. pylori* in both solid and liquid media, the impact of successive subcultivation on cell-surface glycans (LPS and amylose-type glycans) was evaluated. The reference strain NCTC 11637 and the clinical isolates 968 and 14255 were subcultivated in CBA. The impact of successive subcultivation on cell-surface glycans was determined by the analysis of the sugars carried out on the freeze-dried biomass of intact cells. The total LPS (Fig. 5A), given by the amount of Fuc, Glc, Gal, GlcNAc, and Hep, LPS *O*-chain (Fig. 5C), given by the amount of Fuc, Gal, and GlcNAc, were quantified independently based on the output from sugar analysis. The results are expressed in terms of concentration (w/w) for six passages in CBA comprising a total of 13 days in solid media. Between- and within-sample variance was estimated by one-way ANOVA for each component (LPS, LPS *O*-chain, and amylose-type glycans) for the three strains during the subcultivation period. *F*-test ($p = .05$) performed on those estimates retrieved that between-measurements variance was more significant than random errors expressed by within-sample variance for all cases.

For LPS (Fig. 5A), an initial increase in the overall amount of the material was observed in all strains, possibly because of an initial adaptation of the bacteria when passing from a vegetative state in the glycerol inoculums to the solid media. However, this tendency is reversed after the third and the fourth passages after which the amount of total LPS expressed decreases.

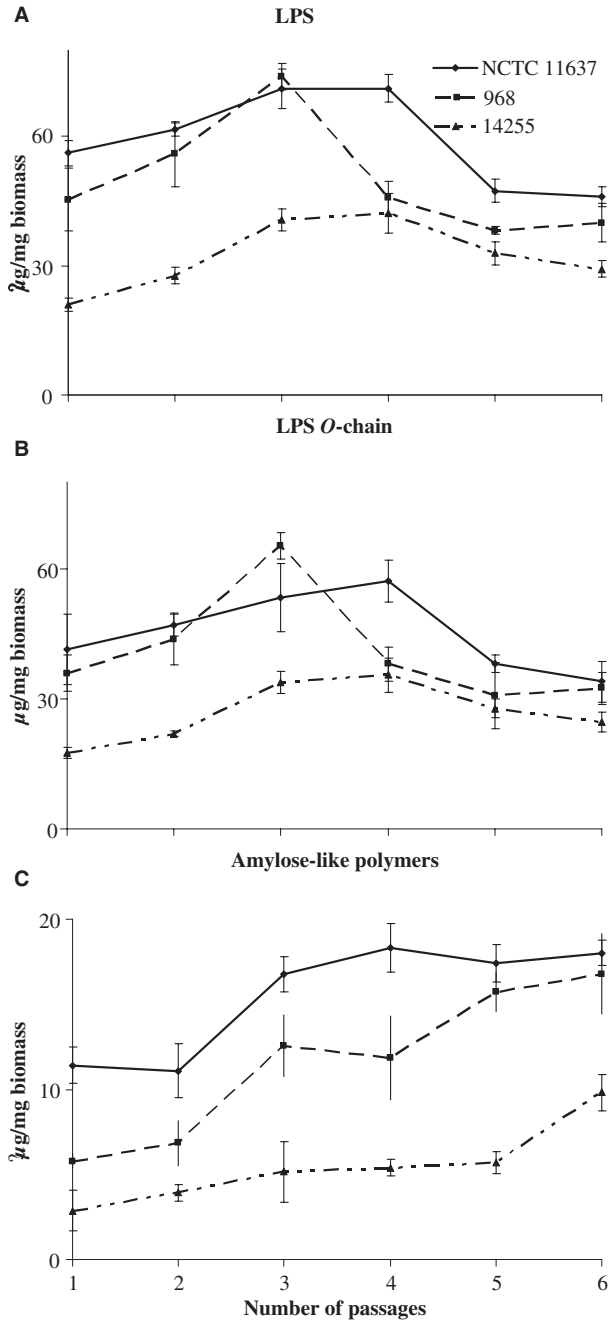

Figure 5 Variation in (*A*) lipopolysaccharides (LPS), (*B*) LPS *O*-chains, and (*C*) amylose-like polymer content in *Helicobacter pylori* cells during subcultivation in Columbia blood agar.

Variations in *O*-chain sugars, presented graphically in Fig. 6B, show the same tendency of the overall LPS. Amylose, in opposition to what has been observed for the LPS, is seen to increase continuously with the incubation time (Fig. 5C). This effect was observed for all strains and demonstrates that *H. pylori* bioaccumulates amylose during the subcultivation period.

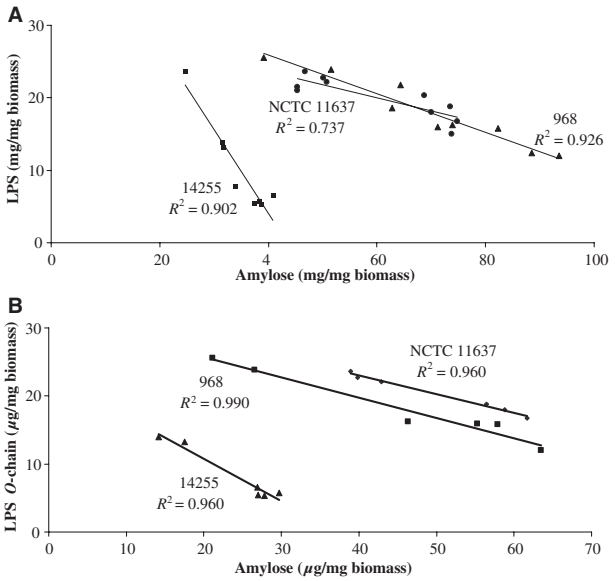

Figure 6 (*A*) Lipopolysaccharides (LPS) versus amylose and (*B*) LPS *O*-chain versus amylose content during subcultivation in Columbia blood agar.

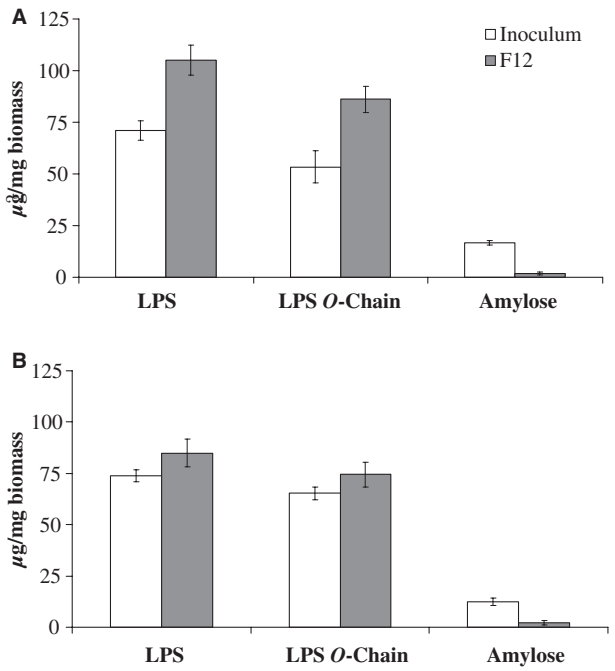

Figure 7 Lipopolysaccharides (LPS), LPS *O*-chains, and amylose content in *Helicobacter pylori* cells present in the initial inoculum and after F12 growth for: (*A*) reference strain NCTC 11 637 and (*B*) clinical isolate 968.

The plot of the total amount of LPS and LPS *O*-chain sugars versus the total amount of amylose throughout the incubation period (Fig. 6) suggests a correlation

between the expression of LPS/LPS *O*-chains and amylose. However, this correlation could only be observed when the data from the first and second passages are not included. This seems to be most likely correlated with an initial adaptation period of the bacteria to solid medium upon recovery from a latent stage resulting from cryostorage.

Amylose Expression in F12 Liquid Media

The *H. pylori* reference strain NCTC 11637 and the clinical isolate 968 obtained after three passages in solid media (day 7) were further recovered to a F12 liquid media.

The glycan profile for each inoculum and F12 material are presented graphically in Fig. 7. In both strains, a statistically significant (Student's *t*-test; $p = .05$) increase was observed in LPS content in the passage from solid to liquid media. This phenomenon was more pronounced in strain NCTC 11637 (48%) than in 968 (12%) and was caused almost exclusively by an increase in the concentration of *O*-chain sugars (62% for NCTC 11637 and 12% for 968). Conversely, the amount of amylose decreased significantly in the passage from solid to liquid media where only residual amounts were observed.

Discussion

In the present work, we describe that *H. pylori* is capable of producing an amylose-like α-(1→4)-D-glucan and further bioaccumulates this polysaccharide under selective pressure induced by subcultivation in the agar medium. Gathered information also suggests that *H. pylori* cell surface is composed of a mixed population of glycans comprising LPS, mannose-rich glycosides as well as capsular/exopolysaccharide α-glucans. The now observed amylose belongs to the class of biomolecules generally referred to as ''bacterial glycogen'' and considered to be a source of readily available glucose [30]. However, unlike most bacteria that indeed produce 8–12% branching in the *O*-6 Glc residues in glycogen [30], in *H. pylori* only vestigial branching was observed (<1%).

Glycogen-like metabolism in bacteria is strongly correlated with the mobilization of ADP-Glc mostly by intracellular synthetic and degradative enzymes belonging to clearly identified families of glycosyltransferases (GT) and of glycosidases/transglycosidases. In a study carried out by Henrissat et al. [31], 55 currently sequenced bacterial genomes were screened for members of the most relevant families of GT (GT5, GT35, GH13, and GH15). The authors concluded that a significant proportion of those microorganisms, including *H. pylori* 26695 and J99 and *Campylobacter jejuni* NCTC 11168, lacked the necessary enzymatic machinery to metabolize glycogen-like molecules. Interestingly, all of them were parasitic, symbiotic, or fastidious thus suggesting that such genotype resulted from a trait associated with the parasitic behavior of the microorganisms. Our results now point in a different direction. Here, it is demonstrated that *H. pylori*, even though being recognized as extremely fastidious, indeed produces α-(1→4)-D-glucans *in vitro*. This finding is in agreement with the recently reported capability of *C. jejuni* 81-176, a virulent model strain used in the study of mechanisms and pathogenesis of *C. jejuni* infection, of also producing a α-(1→4) glucan [32]. Another example of a generally regarded as nonproducing glycogen Gram-negative bacteria is *Neisseria meningitidis* [31,33]. Again, in this case, evidences of strains producing glycogen-like molecules have been presented, as demonstrated by the identification of a genetic *loci* encoding amylosucrase in *N. meningitidis* 93246, responsible for the production of amylose from sucrose [33].

The events triggering the overexpression of α-(1→4)-D-glucans have been correlated with the responses of the organism to environmental pressure or long-term survival strategies. The bioaccumulation phenomenon has been previously observed in microorganisms facing limiting growth conditions [34] as well as in grapevine plants as a nonspecific response to biotic and abiotic stresses [35]. This stress-related strategy based on the production of α-glucan seems to be common to microorganisms and plants. In bacteria, environmental pressure resulting from shortage of a nutrient such as nitrogen in the presence of excess of carbon has also been reported as responsible for the intracellular accumulation of this polysaccharide [31,34,36]. Some bacteria, however, accumulate them in the exponential growth phase [37]. In line with this observation, a recent report concerning the pathogenic Gram-negative bacteria *Vibrio cholerae* states that the capacity of this bacteria to store carbon as glycogen facilitates bacterial fitness when passing both from nutrient-rich human intestinal tract and nutrient-poor aquatic environments, facilitating the transmission to a new host [38]. Thus, it can be inferred that different organisms can trigger at some point of their life cycle the pathways leading to the expression of α-(1→4)-D-glucans as a result of specific environmental pressure.

In this work, aware of *H. pylori* high fastidiousness, we further devoted our attention to fully understanding the influence of environmental pressure resulting from subcultivation in solid agar medium and recovery to liquid medium in the bacterium cell-surface polysaccharides. The results suggest that *H. pylori* cells facing a lag stage resulting from subcultivation in agar medium

bioaccumulate amylose-like polymers over time. The observation of the decrease of cell-surface LPS, namely the *O*-chain, during subcultivation in solid medium is in accordance with previous reports [8,39,40]. A more up-to-date work carried out by Nilson et al. [41] further reinforced that the absence of high-molecular weight *O*-chains was a common feature in two clinical isolates after a number of experimentally passages both in vitro and in mice models. However, this phenotype was not evident in fresh human clinical isolates, where all cells expressed high-molecular weight *O*-chains [41]. In contrast, when *H. pylori* was in an exponential growth phase resulting from cultivation in F12 liquid medium, the amount of expressed amylose-like polymers decreased considerably. This decrease was accompanied by an increase in the overall amount of LPS mainly because of an *O*-chain increment. The observed overexpression of *O*-chain material upon recovery to the liquid medium was in agreement with previous reports [8]. Anyway, F12 has been shown to be able to overcome some of the lower growth rates exhibited by the solid media [6] and to be ideal for the rapid isolation, cultivation, and identification of *H. pylori* from biopsy specimens [42], suggesting its success in mimicking in vivo growth conditions. Furthermore, LPS with high-molecular weight *O*-chains can be considered a characteristic in vivo phenotype during colonization of the human gastric mucosa.

Facing these considerations, it can be stated that phenotypes resulting from F12 growth may resemble in vivo cell-surface patterns. Thus, in vivo cells are led to produce elongated *O*-chains bearing Lewis antigens that are recognized as a prerequisite in the modulation of host–cell interaction and evasion to immune response. Silencing of *O*-chain expression accompanied by bioaccumulation of amylose-like polymers in solid media suggests a redirection in metabolic efforts in response to the no-longer need for interaction with the host and a response to a new environment. Moreover, the expression of elongated *O*-chains might be considered a favorable phenotype among planktonic free-swimming bacteria like the ones encountered during F12 growth. Its substitution by amylose-like polymers upon cultivation in solid medium may be because of a swift toward life on community and formation of biofilm frameworks that render the bacteria protection in certain specific microenvironments.

Based on these considerations, we are also led to think that *H. pylori* cell-surface polysaccharides undergo phase variation in response to different growth stages and environmental pressures. Even though at this stage the biologic role resulting from the decoration of *H. pylori* cell surface with α-glucans is still unclear, the expression of such structures in other bacteria reveals its key importance. Recent studies have highlighted that this class of polysaccharides do not restrict their function just to provide the microorganisms with resources to resist food depletion and long starvation periods. They have been shown to protect against phagocytosis [43] and modulate the host immune response [44,45]. For example, inducing monocytes to differentiate into altered dendritic cells that fail to upregulate CD80 and to present lipid antigens to CD1-restricted T cells, and produce interleukin (IL)-10 but not IL-12 [45]. Thus, the expression/bioaccumulation of α-glucans can be thought to also contribute to the pathogenicity of the bacteria by aiding in the evasion/modulation of immune responses and conferring extra protection against external aggression and unfavorable environments.

Facing all these considerations, further work is being conducted to clarify the biosynthetic pathways behind the expression of α-glucans by *H. pylori*, to clarify the impact of specific environmental factors in amylose bioaccumulation, and to understand its biologic role.

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References

1 Forman D, Graham DY. Impact of *Helicobacter pylori* on society-role for a strategy of ''search and eradicate''. *Aliment Pharmacol Ther* 2004;19:17–21.
2 Ferreira AC, Isomoto H, Moriyama M, Fujioka T, Machado JC, Yamaoka Y. *Helicobacter* and gastric malignancies. *Helicobacter* 2008;13:28–34.
3 Sugiyama T. Development of gastric cancer associated with *Helicobacter pylori* infection. *Cancer Chemother Pharmacol* 2004;54:S12–20.
4 Labigne A, De Reuse H. Determinants of *Helicobacter pylori* pathogenicity. *Infect Agents Dis* 1996;5:191–202.
5 Stevenson TH, Castillo A, Lucia LM, Acuff GR. Growth of *Helicobacter pylori* in various liquid and plating media. *Lett Appl Microbiol* 2000;30:192–6.
6 Testerman TL, McGee DJ, Mobley HLT. *Helicobacter pylori* growth and urease detection in the chemical defined medium Ham's F-12 nutrient mixture. *J Clin Microbiol* 2001;39:3842–50.
7 Azevedo NF, Pacheco AP, Keevil CW, Vieira MJ. Nutrient shock and incubation atmosphere influence the recovery of viable *Helicobacter pylori* from water. *Appl Environ Microbiol* 2004;70:490–3.
8 Monteiro MA. *Helicobacter pylori*: a wolf in sheep's clothing: the glycotype families of *Helicobacter pylori* lipopolysaccharides expressing histo-blood groups: structure, biosynthesis, and

role in pathogenesis. *Adv Carbohydr Chem Biochem* 2001;57: 99–158.
9 Moran AP. Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen *Helicobacter pylori*. *Carbohydr Res* 2008;343:1952–65.
10 Kobayashi M, Lee H, Nakayama J, Fukuda M. Roles of gastric mucin-type *O*-glycans in the pathogenesis of *Helicobacter pylori* infection. *Glycobiology* 2009;19:453–61.
11 Monteiro MA, Michael FST, Rasko DA, Taylor DE, Conlan JW, Chan KH, Logan SM, Appelmelk BJ, Perry MB. *Helicobacter pylori* from asymptomatic hosts expressing heptoglycan but lacking Lewis *O*-chains: Lewis blood-group *O*-chains may play a role in *Helicobacter pylori* induced pathology. *Biochem Cell Biol* 2001;79:449–59.
12 Altman E, Smirnova N, Li J, Aubry A, Logan SM. Occurrence of a nontypable *Helicobacter pylori* strain lacking Lewis blood group *O* antigens and DD-heptoglycan: evidence for the role of the core α-1,6-glucan chain in colonization. *Glycobiology* 2003;13:777–83.
13 Britton S, Papp-Szabo E, Simala-Grant J, Morrison L, Taylor DE, Monteiro MA. A novel *Helicobacter pylori* cell-surface polysaccharide. *Carbohydr Res* 2005;340:1605–11.
14 Monteiro MA, Fulginiti J, Dilts D. Polysaccharides of *Helicobacter pylori*, US patent 7527949.
15 Parodi AJ. Protein glycosylation and its role in protein folding. *Annu Rev Biochem* 2000;69:69–93.
16 Lowe JB, Marth JD. A genetic approach to mammalian glycan function. *Annu Rev Biochem* 2003;72:643–91.
17 Shoaf-Sweeney KD, Hutkins RWA. Adherence, anti-adherence, and oligosaccharides preventing pathogens from sticking to the host. *Adv Food Nutr Res* 2009;55:101–61.
18 Helenius A, Aebi M. *Intracellular functions of N-linked glycans*. *Science* 2001;291:2364–9.
19 Bae K, Choi J, Jang Y, Ahn S, Hur B. Innovative vaccine production technologies: the evolution and value of vaccine production technologies. *Arch Pharm Res* 2009;32:465–80.
20 Erbacher A, Gieseke F, Handgretinger R, Müller I. Dendritic cells: functional aspects of glycosylation and lectins. *Hum Immunol* 2009;70:308–12.
21 Curtis MA, Slaney JM, Aduse-Opoku J. Critical pathways in microbial virulence. *J Clin Periodontol* 2005;32:28–38.
22 Jain A, Gupta Y, Agrawal R, Khare P, Jain SK. Biofilms – a microbial life perspective: a critical review. *Crit Rev Ther Drug Carrier Syst* 2007;24:393–443.
23 Guimarães N, Azevedo NF, Figueiredo C, Keevil CW, Vieira MJ. Development and application of a novel peptide nucleic acid probe for the specific detection of *Helicobacter pylori* in gastric biopsy specimens. *J Clin Microbiol* 2007;45:3089–94.
24 Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol–water and further applications of the procedure. *Methods Carbohydr Chem* 1965;5:83–91.
25 Dubois M, Gilles DA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for the determination of sugars and related substances. *Anal Chem* 1956;8:350–6.
26 Harris PJ, Blakeney AB, Henry RJ, Stone BA. Gas-chromatographic determination of the monosaccharide composition of plant cell wall preparations. *J AOAC Int* 1988;71:272–5.
27 Ciucanu I, Kerek F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* 1984;131:209–17.
28 Aspinall GO, Monteiro MA, Pang H, Walsh EJ, Moran AP. Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the *O* antigen chain and core oligosaccharide regions. *Biochemistry* 1996;35:2489–97.
29 Dourado F, Madureira P, Carvalho V, Coelho R, Coimbra MA, Vilanova M, Mota M, Gama FM. Purification, structure and immunobiological activity of an arabinan-rich pectic polysaccharide from the cell walls of *Prunus dulcis* seeds. *Carbohydr Res* 2004;339:2555–256.
30 Ball SG, Morell MK. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annu Rev Plant Biol* 2003;54:207–33.
31 Henrissat B, Deleury E, Coutinho PM. Glycogen metabolism loss: a common marker of parasitic behaviour in bacteria? *Trends Genet* 2002;18:437–40.
32 Papp-Szabó E, Kanipes MI, Guerry P, Monteiro MA. Cell-surface alpha-glucan in *Campylobacter jejuni* 81-176. *Carbohydr Res* 2005;340:2218–21.
33 Zhu P, Tsang RSW, Tsay CM. Nonencapsulated *Neisseria meningitidis* strain produces amylopectin from sucrose: altering the concept for differentiation between *N. meningitidis* and *N. polysaccharea*. *J Clin Microbiol* 2003;41:273–8.
34 Preiss J. Regulation of glycogen synthesis. In: Neidhart FC, Curtiss R, eds. *Escherichia coli* and *Salmonella* – Cellular and Molecular Biology. Washington, DC: American Society for Microbiology, 1996; 1015–24.
35 Oliveira H, Barros AS, Delgadillo I, Coimbra MA, Santos C. Effects of fungus inoculation and salt stress on physiology and biochemistry of in vitro grapevines: emphasis on sugar composition changes by FT-IR analyses. *Environ Exp Bot* 2009;65:1–10.
36 Yang H. Coordinate genetic regulation of glycogen catabolism and biosynthesis in *Escherichia coli* via the CsrA gene product. *J Bacteriol* 1996;178:1012–101.
37 Preiss J, Romeo T. Physiology, biochemistry and genetics of bacterial glycogens synthesis. *Adv Microb Physiol* 1989;30:183–238.
38 Bourassa L, Camilli A. Glycogen contributes to the environmental persistence and transmission of *Vibrio cholerae*. *Mol Microbiol* 2009;72:124–38.
39 Moran AP, Helander IM, Kosunen TU. Compositional analysis of *Helicobacter pylori* rough-form lipopolysaccharides. *J Bacteriol* 1992;174:1370–7.
40 Walsh EJ, Moran AP. Influence of medium composition on the growth and antigen expression of *Helicobacter pylori*. *J Appl Microbiol* 1997;83:67–75.
41 Nilson C, Skoglund A, Moran AP, Annuk H, Engstrand L, Normark S. Lipopolysaccharide diversity evolving in *Helicobacter pylori* communities through genetic modifications in fucosyltransferases. *PLoS ONE* 2008;3:1–14.
42 Sainsus N, Cattori V, Lepadatu C, Hofmann-Lehmann R. Liquid culture medium for the rapid cultivation of *Helicobacter pylori* from biopsy specimens. *Eur J Clin Microbiol Infect Dis* 2008;27:1209–17.
43 Stokes RW, Norris-Jones R, Brooks DE, Beveridge TJ, Doxsee D, Thorson LM. The glycan-rich outer layer of the wall of *Mycobacterium tuberculosis* acts as an antiphagocity capsule limiting the association of the bacterium with macrophages. *Infect Immun* 2004;72:5676–86.
44 Bittencourt VCB, Figueiredo RT, da Silva RB, Mourão-Sa DS, Fernandez PL, Sassaki GL. An α-glucan of *Pseudallesscheria boydii* is involved in fungal phagocytosis and Toll-like receptor activation. *J Biol Chem* 2006;281:22614–23.
45 Gagliardi MC, Lemassu A, Teloni R, Marriotti S, Sargentini V, Pardini M. Cell wall-associated alpha-glucan is instrumental for *Mycobacterium tuberculosis* to block CD1 molecule expression and disable the function of dendritic cells from infected monocyte. *Cell Microbiol* 2007;9:2081–92.

