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In vitro binding of *Helicobacter pylori* to monohexosylceramides

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***H. pylori* is the major cause of human gastritis, duodenal ulcer and thus gastric adenocarcinoma. Many glycosphingolipid species have been postulated as receptors for *H. pylori* and it is likely that *H. pylori* attachment requires multiple, perhaps sequential receptor/ligand interactions. In this study, the binding of a number of *H. pylori* clinical isolates, as well as stock strains, to acid and neutral glycosphingolipids separated on thin-layer chromatograms was characterized under microaerobic conditions. All *H. pylori* clinical isolates, laboratory strains and type culture collection strains recognized galactosylceramide (Galβ1Cer) with ceramide containing sphingosine and hydroxylated fatty acid (type I), or non-hydroxylated fatty acid (type II), on thin-layer chromatograms and when incorporated into liposomes. The clinical isolates bound stronger to Galβ1Cer (type II) than Galβ1Cer (type I) on TLC, whereas lab and culture collection strains showed the opposite binding preference. A clear preference in binding to Galβ1Cer (type I) incorporated into liposome was shown by most tested strains. Clinical isolates bound well to glucosylceramide (Glcβ1Cer) with hydroxylated fatty acid, whereas weak binding to this glycolipid was detected with the lab and type collection strains. None of the tested strains bound Glcβ1Cer with non-hydroxylated fatty acid on the solid surface, but some strains of both clinical or type collection origins showed weak or very weak binding in the liposome assay. A clear distinction between the binding specificity of living organisms (under microaerobic conditions) as opposed to dying organisms (under normoxic conditions) illustrates the importance of cellular physiology in this process. These studies illustrate lipid modulation of the potential receptor function of monohexosylceramides and the distinction between the receptor repertoire of *H. pylori* clinical isolates and cultured strains commonly used to study host-cell adhesion.**

Keywords: glycolipid receptors, fatty acid aglycone, laboratory vs. clinical strains

Introduction

H. pylori is a spiral-shaped highly motile gram negative organism, which colonizes human stomach, the primary etiology of chronic active or type B gastritis [1,2], duodenal ulcer [3], gastric carcinoma [4], and mucosa-associated lymphoid tumors [5,6]. This bacterium hemagglutinates erythrocytes of many species [7,8], and was also found to bind receptors on different tissue culture cells [9]. Binding of *H. pylori* to gastrointestinal epithelial cells HuTu was shown to be inhibited by sialyllactose, a sialic acid-containing oligosaccharide [10]. Internalization of the bacterium by HEp-2 cells indicated that the binding was determined by an N-acetylsialyllactose-binding adhesin and other factors expressed by the living cells [11]. The gene *hpa* A, which encodes the sialyllactose binding fibrillar hemagglutinin, was cloned and sequenced [12]. *H. pylori* specifically recognized sialic acid containing glycolipids [13], together with Gg_3 and Gg_4 glycolipids [14,15], phosphatidylethanolamine [16], and the Lewis^b antigen [17]. The adhesin responsible Le^b binding has been identified [18] and shown to play an important role in an animal model [19]. A general role in attachment in humans is more controversial [20,21] but may be associated with more severe clinical outcome [22]. It has also been reported that *H. pylori* bound to acid-glycosphingolipids GM3 [8,23], sialylpolyglycosylceramides [24], specific lipid isoforms of lactosylceramide [25] and to sulfatide [26,27], particularly after stress [28]. Surface located hsp70 was implicated in this latter binding and the recombinant *H. pylori* hsp70 has been since shown to bind sulfatide [29]. This repertoire of adhesin/receptor interactions likely acts in combinations, variable at

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different growth stages, to define the risk factors for colonization and subsequent disease [30].

Strain differences and binding assays under non-physiological conditions in part, explain these differences, but it is likely that *H. pylori*-host cell binding is multifaceted [31].

We now demonstrate the binding of laboratory cultured strains and clinical isolates of *H. pylori* to various forms of ceramide monohexosides on thin-layer chromatograms (TLC) and incorporated into liposomes. The difference in the binding selectivity of these two groups of *H. pylori* strains, highlights the need for caution in the interpretation of binding for *in vitro* adapted organisms, and the significant effect of microaerobic conditions questions the significance of binding under non-physiological conditions.

Material and methods

Bacterial strains and cultivation conditions

We have tested 10 clinical strains isolated from patients at HSC: 95, 431, 475, 576, 782, 832, 877, 909, 949 and 955. In addition, R strain (rough), a strain with incomplete LPS, laboratory strain LC11 with semi-rough LPS and type culture collection strains CCUG 17874 and CCUG 17875 with incomplete LPS from Culture Collection of Göteborg University (CCUG) were used in this study.

Bacteria were cultured on soft (1%) Brucella agar (BA) (Difco Lab., Detroit, MI), supplemented with 10% heat-inactivated fetal calf serum (GibcoBRL) and enriched with 0.1% (v/v) IsoVitaleX (BBL Enrichment, Cockeysville, MD, USA). The bacterial strains were maintained, after isolation from patients on 5% sheep-blood Columbia agar, in Tryptic Soy broth (Oxoid, Nepean, Ontario, Canada), containing 20% (v/v)glycerol at -70°C. Three-day old bacterial growth was used to inoculate fresh BA plates, which were incubated at 37°C under microaerobic conditions for 3 days. Bacterial growth was then washed twice in TBS (Tris buffer saline pH 7.4) and resuspended in the same buffer to a final concentration of 1×10^9 CFU prior to binding assay.

Lipids and glycosphingolipids

The glycosphingolipids used in this study are listed in Tables 1 and 2. Hydroxylated ceramide, Galβ1Cer type I (ceramide containing hydroxylated fatty acid), Galβ1Cer type II (ceramide containing non-hydroxylated fatty acid), non-hydroxylated Glcβ1Cer, from human (Gaucher's) spleen, sulfatide (SGC) and gangliosides GM3, GM1, GD3, GD1a and GD1b were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Sulfogalactosylglycerolipid (SGG) was prepared from bovine testes as previously described [32]. Glcβ1Cer with hydroxylated fatty acid was purchased from Matreya Inc. (Brockville, Ontario, Canada). Glucosylceramide (Glcβ1Cer), lactosylceramide, Globotriaosylceramide (Gb_3) and globotetraosylceramide (Gb_4) were isolated from human kidneys [33]. Gangliotriaosylceramide (Gg_3) was prepared from guinea pig blood [34], while gangliotetraosylceramide (Gg_4) was obtained by desialylation of GM1 [16].

TLC binding assay

The binding of *H. pylori* to glycolipids on thin-layer chromatogram was carried out as described elsewhere [16,35]. TLC plates were prepared in duplicate, using 5 µg of purified or 10 µg of glycolipid mixtures per lane. The glycolipids were separated on plastic-backed silica gel (polygram SilG, Duren, Germany), using chloroform:methanol:water, 60:35:8 per volume as a solvent system. One TLC plate was stained with anisaldehyde [36], while a second plate was blocked in 3% (wt/vol) gelatin for 2 hours at 37°C and washed in TBS. The washed TLC plate was incubated with bacteria (1×10^9) CFU in TBS for 2 hours at 37°C under microaerobic conditions. After 5 washes with TBS to remove unbound bacteria, the chromatograms were incubated for 2 h at room temperature (RT) or overnight at 4°C with anti-*H. pylori* rabbit polyclonal antisera raised against whole bacteria. After washing, the TLC plates were incubated with goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, Mississuga, Ontario, Canada) for 1 hour at RT. The chromatograms were then washed again and the conjugate-bound peroxidase was detected with 4-chloro-naphthol and hydrogen peroxide (Sigma). After the bands had developed the plates were dried at RT in the dark, and photographed. Tlc overlays for each strain were repeated at least 10 times.

Liposome assay

Unilamellar liposomes were prepared using "reverse phase evaporation" method as previously reported [37]. Briefly, 20 µmol of lipids (glycolipid/egg phosphatidylglycerol/cholesterol/phosphatidylcholine 0.2/0.5/10/10 (mol/mol) in chloroform were dried under nitrogen and redissolved in 15 µl chloroform:diethyl ether (1:1, v:v), thereafter 4 ml PBS was added and sonicated for 3 min at room temperature (RT) using a sonicator bath. The liposomes were formed after evaporation of the organic phase from the emulsified solution using rotary evaporator. Aggregation of bacteria was carried out in a microtiter plate by adding one part liposome suspension to two parts of bacterial suspension (1×10^9 CFU), mixed well and the plates were incubated at RT for 1 hour. Evaluation of bacterial aggregation was carried out using phase contrast light microscopy.

Preparation of anti-*H. pylori* polyclonal antibodies

Antibodies raised against whole *H. pylori* were obtained as earlier reported [38]. Briefly, a whole polyvalent antibody against *H. pylori* was raised by intravenous injection of New Zealand white rabbit (about 1500 g) with formalized *H. pylori* bacteria. Bacterial suspension (0.5 ml) with a final concentration of (2×10^4 per ml) in phosphate-buffered saline was used

Table 1. Binding of various *H. pylori* strains to glycolipids and phospholipids separated on thin-layer chromatogram

Glyco- or sphingolipid structures (Trivial name)	*Binding strain*										
	LC11[1]	*95*	*431*	*475*	*576*	*782*	*832*	*877*	*909*	*949*	*955*
Neutral glycolipids											
1. Ceramide[3]	−	−	−	−	−	−	−	−	−	−	−
2. Glcβ1Cer (Glucosylceramide)[3]	+/−[4]	+	+	+	+	−	+	+	+	+	+
3. Galβ1Cer (Galactosylceramide)[3]	+/−	+	+	+	+	+	+	+	+	+	+
4. Galβ1-4Glcβ1Cer (Lactosylceramide)	+	+	+	+	+	+	+	+	+	+	+
5. Galα1-4Galβ1-4Glcβ1Cer (Globotriaosylceramide)	−	−	−	−	−	−	−	−	−	−	−
6. GalNAcβ1-3Galα1-Galβ1-4Glcβ1Cer (Globotetraosylceramide)	−	−	−	−	−	−	−	−	−	−	−
7. GalNAcβ1-4Galβ1-4Glcβ1Cer (Gangliotriaosylceramide)	++	++	++	++	++	++	++	++	++	++	++
8. Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1Cer (Gangliotetraosylceramide)	++	++	++	++	++	++	++	++	++	++	++
Acid glycolipids											
9. NeuAcα2-3Galβ1-4Glcβ1Cer (NeuAc-GM3)	+/−[4]	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
10. Galβ1-3GalNAβ1-4(NeuAcα23) Galβ1-4Glcβ1Cer (NeuAcGM1)	−	−	−	−	−	−	−	−	−	−	−
11. NeuAcα2-8NeuAcα2-3Galβ1-4 Glcβ1Cer (GD3)	−	−	−	−	−	−	−	−	−	−	−
12. NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1Cer(GD1a)	−	−	−	−	−	−	−	−	−	−	−
13. Galβ1-3GalNAcβ14(NeuAcα2-8NeuAcα2-3) Galβ1-4Glcβ1Cer (GD1b)	−	−	−	−	−	−	−	−	−	−	−
Phospholipids											
14. Phosphatidylethanolamine (PE)	+	+	+	+	+	+	+	+	+	+	+
15. Phosphatidylserine (PS)	−	−	−	−	−	−	−	−	−	−	−
16. Phosphatidylcholine (PC)	−	−	−	−	−	−	−	−	−	−	−

[1]Lab strain semi-rough LPS; [2]R: Rough strain indicating incomplete LPS; [3]Ceramide with hydroxylated fatty acid; [4]+/− variable binding.

to inject the rabbits, followed by injections (0.5 ml of 2×10^8 bacteria per ml) on 2 and 6 weeks. The presence of polyclonal antibody was detected using immunoblots of whole cell sonicates of various *H. pylori* strains.

Results

Binding of various *H. pylori* strains to acid and neutral glycolipids

Binding of different *H. pylori* strains to acid and neutral glycolipids by overlay is summarized in Table 1. All strains including rough (R) and lab (LC11) strains recognized Glcβ1Cer with ceramide containing hydroxylated fatty acid, except strain 782 (no. 2 Table 1). Substitution of the glucose with β-galactose in 4-position (lactosylceramide, LacCer) changed the binding pattern of *H. pylori* strains. All strains except R strain bound LacCer (no. 4). Furthermore, substitution of the terminal galactose of LacCer by α-galactose in 4-position (Gb_3, no. 5) or α-linked sialic acid in 3-position (GM3 ganglioside, no. 11) or by α-GalNAcβl-3Gal in 4-position (Gb_4, no. 6) resulted in loss of bacterial binding. On the other hand, substitution of terminal galactose by β-galactosamine in 4-position of LacCer (Gg_3, no. 7) increased the binding relative to the diglycosylceramide by all bacterial strains. An additional substitution of Gg_3 by β-galactose in 3-position (Gg_4, no. 8) was tolerated by those strains. Galactosylceramide with hydroxylated fatty acid (GalCer, no. 3) was recognized by *H. pylori* strains. None of the tested strains of *H. pylori* showed binding to any of the gangliosides GM1, GD3, GD1a and GD1b (nos. 10, 11, 12 and 13 of Table 1) separated on TLC plates. Among three phospholipids tested, only PE was recognized by *H. pylori* strains.

Influence of culture period on the binding of *H. pylori*

In Figure 1, binding of *H. pylori* clinical isolate 431 to glucosylceramide (lane 2), hydroxy galactosylceramide (lane 3), and lactosylceramide (lanes 2 and 3), was stable

Table 2. Binding of *H. pylori* to glucosylceramide and galactosylceramide containing hydroxylated or non-hydroxylated fatty acids on thin-later chromatograms

	Glycolipid			
H. pylori strain	*GalCer (OH)*	*GalCer (non-OH)*	*GlcCer (OH)*	*GlcCer (non-OH)*
LC11	+/−	+/−	+/−	−
95	+	++	++	−
431	+	++	++	−
475	+	++	++	−
576	+	++	++	−
782	+	++	+	−
832	+	++	++	−
877	+	++	++	−
909	+	++	++	−
949	+	++	++	−
955	+	++	++	−
CCUG 17874	++	(+)/−	+	−
CCUG 17875	++	(+)/−	+	−
R	++	(+)/−	+/−	−

++: strong binding; +: weak binding; +/−: variable binding; (+)/−: weak & variable binding; and −: negative binding.

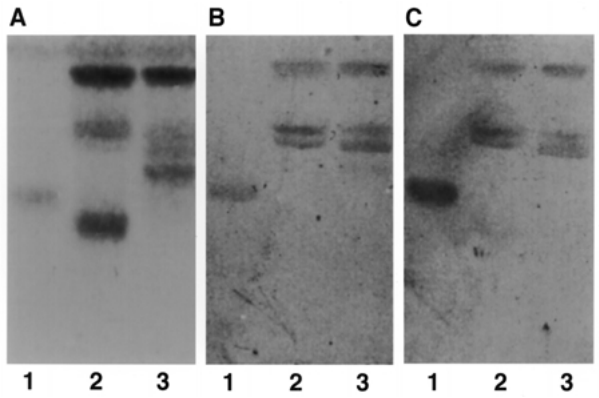

Figure 1. Binding of *H. pylori* to galactosylceramide and glucosylceramide on thin-layer chromatogram (TLC). TLC plates stained with anisaldehyde (A) or by overlay assay with *H. pylori* clinical isolate 431 grown for 3 days (B) and grown for 10 days (C). The glycolipids were separated on plastic-backed polygram SilG plates using chloroform:methanol:water 60:35:8, as solvent system. The lanes contain the following glycolipids: (1) gangliotriaosylceramide, 5 μg; (2) a mixture of neutral glycolipids containing (from top to bottom) galactosylceramide, lactosylceramide and globotetraosylceramide, 10 μg, from human kidney; (3) crude neutral fraction containing (from top to bottom) glucosylceramide and ceramide dihexoside(runs as a triplet), 10 μg, from human placenta. *H. pylori* binds GalCer, GlcCer and the LacCer doublet (third band in CDH region not recognized).

and reproducible regardless of whether the bacteria were grown for 3 or 10 days. In the same manner, no binding to Gb_4 (lane 2) was detected, whereas Gg_3 (lane 1) was strongly bound by the bacterium after culture for 10 days (lane 1 of Figure 1C) in compansion with 3-day grown bacteria (lane 1 of Figure 1B).

The role of hydroxylated fatty acid on the binding of *H. pylori* to monohexosylceramides

Figures 2 and 3, show clear and specific tlc binding of all tested *H. pylori* strains to GalβCer(OH), GalβCer(non-OH) and GlcβCer(OH). The results are summarized in Table 2. The lab and type collection strains as well as R strain, demonstrated a clear preference in binding to GalβCer(OH), and

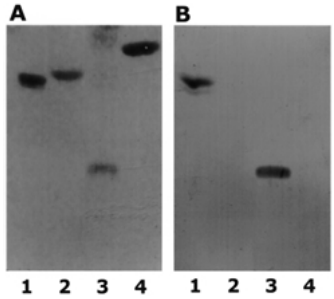

Figure 2. Binding of *H. pylori* to glucosylceramide with and without hydroxylated fatty acids. TLC plates after staining with anisaldehyde (A) or after binding with *H. pylori* clinical isolate 431 (B). The glycolipids were separated on plastic-backed polygram SilG plates using chloroform:methanol:water 60:35:8, as solvent system. The lanes contain the following glycolipids: (1) glucosylceramide (hydroxylated fatty acid), 5 μg; (2) glucosylceramide (non-hydroxylated fatty acid), 5 μg; (3) gangliotriaosylceramide, 5 μg; and (4) ceramide (hydoxylated fatty acid), 5 μg.

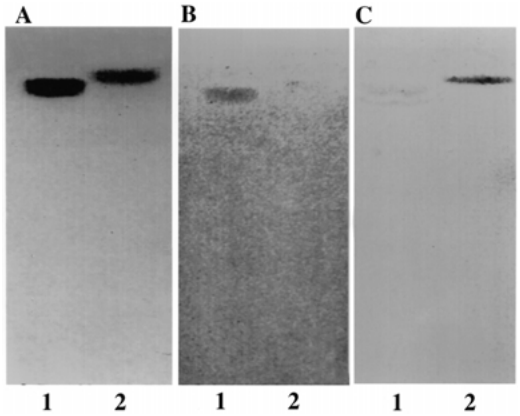

Figure 3. Binding of *H. pylori* to galactosylceramide with and without hydroxylated fatty acids. TLC plates stained with anisaldehyde (A) or after binding with *H. pylori* rough strain R (B) or after binding with *H. pylori* clinical isolate 431 (C). The glycolipids were separated on plastic-backed polygram Si1G plates using chloroform:methanol:water 60:35:8, as solvent system. The lanes contain the following glycolipids: (1) galactosylceramide with hydroxylated fatty acid, 5 μg; (2) galactosylceramide with non-hydroxylated fatty acid 5 μg.

weaker to GalβCer(non-OH). In contrast, the clinical isolates bound strongly to GalβCer(non-OH) and weakly to GalβCer(-OH). Glucosylceramide with hydroxy fatty acid, GlcβCer(-OH), was bound by all *H. pylori* strains, however, the lab and type strains only bound this GSL weakly. None of the tested strains bound GlcβCer with non-hydroxylated fatty acid.

H. pylori binding to monohexosyl ceramide containing liposomes

To determine whether the membrane bilayer may affect the epitope presentation of mono-hexosylceramides for binding, we tested lab, clinical and type collection strains in a liposome aggregation assay. Bacterial binding was assessed by extent and size of aggregates using phase-contrast microscopy. Representative data for the rough strain are shown in Figure 4. Strong binding to GalCer(OH) and GlcCer(OH) liposomes was seen (Figure 4A, D respectively), somewhat less for GalCer(non-OH) (Figure 4B. Little aggregation was detected when the same strain was incubated with GlcCer(non-OH) liposomes (Figure 4E), No bacterial aggregates of hydroxy ceramide liposomes (Figure 4C) or control liposomes without glycolipid (Figure 4F) were observed. The overall liposomal binding results for all strains are presented in Table 3. Strong aggregation with GalβCer(non-OH) liposomes was obtained when clinical isolates and type strains were used, whereas R and LC11 showed weak binding. Interestingly, the lab and collection strains induced strong aggregations with GalβCer(OH) incorporated into liposomes confirming their binding preference to hydroxylated galactosylceramide. GlcβCer(OH) liposomes were strongly bound by lab, rough and clinical isolates, except strain 95. However, this GSL containing liposome was poorly recognized by type collection strain CCUG 17874. Strains 95, 909, CCUG 17875 and CCUG 17874 caused weak and very weak liposome aggregation when incubated with GlcβCer(non-OH) containing liposomes.

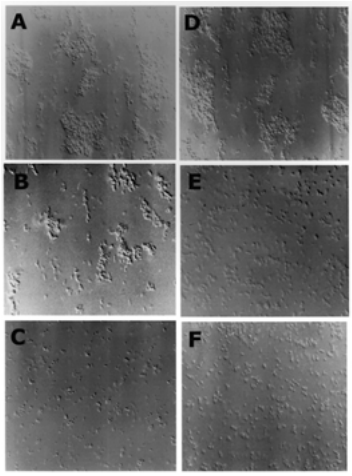

Figure 4. *H. pylori* liposome aggregation assay R strain was assayed for liposomal aggregation. Liposomes: Galβ1Cer (OH) (A), Galβ1Cer(non-OH) (B), ceramide(OH) (C), Glcβ1Cer (OH) (D), Glcβ1Cer (non-OH), and liposomes without glycolipids (F).

Table 3. Binding of *H. pylori* to glucosylceramide and galactosylceramide containing hydroxylated or non-hydroxylated fatty acids incorporated into liposomes

	Glycolipid			
H. pylori strain	*GalCer (OH)*	*GalCer (non-OH)*	*GlcCer (OH)*	*GlcCer (non-OH)*
LC11	++	+	++	−
095	+	++	−	(+)
832	+	++	++	−
909	+	++	++	(+)
955	+	++	++	−
CCUG 17874	++	++	+	(+)
CCUG 17875	++	++	++	+
R	++	+	++	−

++: strong binding (strong aggregation); +: weak binding; (+): very weak binding; −: negative binding.

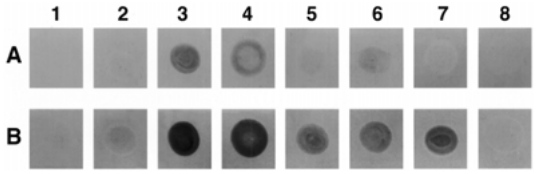

Figure 5. Influence *of* microaerobic conditions and temperature on the lipid binding of *H. pylori*. Two identical TLC plates of glycolipids (3 μg) were overlayed with *H. pylori* R strain. One plate was incubated under normal atmospheric conditions at RT (A), whereas the second plate was incubated under microaerobic conditions at 37°C (B). glycolipids: (1) GlcCer (non-OH); (2) GlcCer(OH); (3) Gg_3; (4) Gg_4; (5) GalCer(OH); (6) GalCer (non-OH); (7) PE and (8) Ceramide (OH).

Effect of microaerobic environment on *H. pylori* binding to glycolipids

Binding of *H. pylori* CCUG 17874 strain to glycolipids separated on thin layer chromatograms was increased when the binding assay was carried out at 37°C and under microaerobic conditions. Binding of bacteria to GlcβCer(OH) and PE was observed only when microaerobic conditions and 37°C were used. A clear increase in binding was detected for Gg_3, Gg_4 and GalβCer(non-OH). No binding was detected to GlcβCer (non-OH) or ceramide under either assay conditions. We have previously shown *H. pylori* to bind PE at room temperature under microaerobic conditions [38]. Therefore the difference

in binding is a result of the atmospheric change rather than temperature.

Discussion

H. pylori is associated with human gastric diseases, gastric ulcer and gastric adenocarcinoma [39,40]. One of the important pathogenic factors in colonization and infection of the bacterium is the capacity to recognize specific receptors in the stomach. Many such species have been proposed but the role of each *in vivo* still remains speculative [21,31].

The binding specificity of *H. pylori* strains for ceramide monohexosides using a solid-surface tlc overlay assay correlated well with agglutination of liposomes, a model for bilayer membranes. Our studies indicate that this specificity is modulated by repeated *in vitro* passage.

These results contrast an earlier report regarding LacCer binding, where no binding of *H. pylori* to monohexosylceramides was detected [25]. This might be based on the differences in the conditions for bacterial cultivation and binding assay. However, the greater overall recognition of GalβCer in our study indicates the preference for this lipid-sugar linkage. Furthermore, GlcβCer binding was seen primarily in the clinical isolates, not tested in the study of Ångström *et al.* The binding of *H. pylori* strains to GlcβCer and LacCer glycolipids was similar, which may indicate that the bacteria recognized the internal glucose moiety of the Galβ4Glcβ1Cer. The galactose linked at the 4-position of glucose was tolerated but probably not part of the binding epitope.

Our finding that substitution at the gal 4 OH of LacCer by α-galactose prevented binding whereas βN-acetyl galactosamine substitution increased binding, suggests Gg_3 provides a new binding epitope—GalNAcβ1-3Gal, recognized by other bacterial pathogens [41]. The increased binding to Gg_3 after a longer period of *in vitro* culture could be related to the expression of this adhesin at stationary phase, rather than within early stages of *H. pylori* growth.

H. pylori is a microaerophilic organism and we have suggested that to maintain physiological relevance, binding assays should be performed under such conditions [16,28,38,42]. Though many receptor species have been identified [3l], most studies have not conformed to this tenet. We now clearly show a marked difference between the aerobic and microaerobic binding phenotype. In particular PE binding, which we have proposed to mediate the attachment of several bacteria [43–45], is only seen under physiological, microaerobic conditions.

Using these conditions, the present report demonstrates new receptor-active glycolipids for *H. pylori*, namely Galβ1Cer and Glcβ1Cer, both of which are closely apposed to the membrane bilayer. Using both thin-layer chromatogram overlay and liposomal aggregation assays, all strains tested showed positive binding to galactosyl ceramide with hydroxylated or non-hydroxylated fatty acid residues in the ceramide. Both the laboratory LC11 and R strains, as well as the two type collection strains, showed weak and variable binding to GalβCer with non-hydroxylated fatty acids. In contrast, the same strains showed a stronger binding profile to GalβCer-OH than the wild type strains. Since such extensively cultured strains are often deficient in the O-side chain of LPS [46], this could implicate LPS in the interaction with the receptor. This could be a direct role mediated by carbohydrate–carbohydrate interaction [30] or alternatively, incomplete LPS might expose or inhibit an adhesin. Hence, there could be two important factors for optimal interaction: the binding epitope presentation of GalβCer and the expression of LPS.

Hydrogen bonds between amide NH and the oxygens of the hydroxyl group of the fatty acid and glycosidic linkage may change the sugar ring to a position pointing away from ceramide, giving a shovel-shape or L-shape with low energy conformer [25,47] to allow discrimination of the hydroxylated species.

The crystal analysis of GalCer [47] shows that the plane of sugar ring is turned almost parallel to the layer interface. In the hydroxylated form of Galβ1Cer, the galactose head group forms an extensive lateral network due to hydrogen bonds of the amide N-H group directed towards the oxygens of the glycosidic linkage and the hydroxyl group of the fatty acid within the polar region of each layer demonstrating an L- or shovel-shape conformation. The lipid environment could have a major effect on monohexosyl ceramide binding. Molecular mechanics calculations for glucosyl ceramide containing hydroxy and normal fatty acids [48] revealed that the GlcβCer could potentially exist in nine different conformers, the relative frequency of which, would be affected by the relative plane of the lipid bilayer. The composition of the lipid moiety of a GSL could well affect the relative plane of the bilayer to alter the frequency of such conformers. Angstrom *et al.* [25] demonstrated that low energy conformers based on the hydrogen bonding between 2-D hydroxyl group of the fatty acid and 2-OH or 6-OH of the glucose ring were preferred for *H. pylori* binding to LacCer. This is consistent with our observed binding preference for hydroxylated as opposed to non-hydroxylated GlcβCer. The only difference between GlcβCer and GalβCer is the configuration of the 4-O, which must therefore be critical for bacterial binding to the hydroxylated fatty acid containing species. The equatorial 4'OH in GlcCer allows binding when the anomeric linkage is restricted by H-bonding to the 2-D fatty acid hydroxyl (and similarly in hydroxylated LacCer). The axial position in GalCer does not require this restriction to allow binding.

The aglycone fatty acid was implicated in the binding of propioni bacteria to LacCer, [49] prior to the studies on *H. pylori* [25]. The *E. coli* K88 adhesin provides both a precedent for ceramide monohexoside and aglycone modulation since hydroxylated (but not nonhydroxylated) fatty acid containing GalβCer was recognized. In this case GlcβCer was not bound

[50,51]. In contrast *Actinobacillus pleuropneumoniae,* binds both forms of GalβCer and GlcβCer [52].

The expression of incomplete LPS by rough and laboratory strains and complete LPS by clinical isolates might explain the difference in binding of those strains to GalβCer or may be only a marker of laboratory adaptation. However, LPS/GSL binding has been demonstrated [52]. Our study also illustrates the potential danger of restricting *in vitro* analysis to bacterial strains which have undergone long term laboratory passage. The consistence of the binding data for the clinical strains suggests that binding of *H. pylori* to ceramide monohexoside may play an important role in the host colonization and pathogenesis of these organisms, since these GSL are so closely apposed to the membrane bilayer.

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