α1,4GlcNAc-capped mucin-type O-glycan inhibits cholesterol α-glucosyltransferase from Helicobacter pylori and suppresses H. pylori growth

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Helicobacter pylori infects over half of the world’s population and is thought to be a leading cause of gastric ulcer, gastric carcinoma, and gastric malignant lymphoma of mucosa-associated lymphoid tissue type. Previously, we reported that a gland mucin (MUC6) present in the lower portion of the gastric mucosa containing α1,4-N-acetylglucosamine (α1,4GlcNAc)-capped core 2-branched O-glycans suppresses H. pylori growth by inhibiting the synthesis of α-glucosyl cholesterol, a major constituent of the H. pylori cell wall (Kawakubo et al. 2004. Science. 305:1003-1006). Therefore, we cloned the genomic DNA encoding cholesterol α-glucosyltransferase (HP0421) and expressed its soluble form in Escherichia coli. Using this soluble HP0421, we show herein that HP0421 sequentially acts on uridine diphosphoglucose and cholesterol in an ordered Bi-Bi manner. We found that competitive inhibition of HP0421 by α1,4GlcNAc-capped core 2-branched O-glycan is much more efficient than noncompetitive inhibition by newly synthesized α-glucosyl cholesterol. Utilizing synthetic oligosaccharides, α-glucosyl cholesterol, and monosaccharides, we found that α1,4GlcNAc-capped core 2-branched O-glycan most efficiently inhibits H. pylori growth. These findings together indicate that α1,4GlcNAc-capped O-glycans suppress H. pylori growth by inhibiting HP0421, and that α1,4GlcNAc-capped core 2 O-glycans may be useful to treat patients infected with H. pylori.

Keywords: α4GlcNAc-capped core 2-branched O-glycan/cholesterol α-glucosyltransferase/growth inhibition/Helicobacter pylori/novel antibiotics

Introduction

Helicobacter pylori infects over half of the world’s population and is a leading cause of gastric ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue-type malignant lymphoma (Marshall and Warren 1984; Peek and Blaser 2002). This infection is ubiquitous among human population, and recent studies show that an association of H. pylori with humans took place before modern humans spread out from Africa. H. pylori apparently spread from East Africa around 58,000 years ago (Linz et al. 2007). Before then, the prototypic microbe apparently jumped from humans to wild cats such as the cheetah approximately 200,000 years ago (Eppinger et al. 2006).

In H. pylori infection, bacteria first attach to surface mucous cells of the gastric mucosa, an attachment facilitated by binding of the H. pylori adhesion molecule Bab A to blood group antigens expressed on mucous cells (Iver et al. 1998). The human population of South American Amerindians dominantly express blood group O antigen (Mourant et al. 1976). Interestingly, Bab A from this population binds blood group O antigen more efficiently than other blood group antigens (Aspholm-Hurtig et al. 2004).

Once H. pylori infects the stomach, the microbe preferentially colonizes the surface mucous cell-derived mucins (Hidaka et al. 2001). This infection initiates an inflammatory response in infected epithelia and is associated with mucosal infiltration of neutrophils, monocytes, and lymphocytes, causing gastritis (Rauws et al. 1988). If untreated, the disease further progresses to gastric ulcer, carcinoma, and malignant lymphoma.

The second phase of the disease progression, however, occurs in 3–6% of infected individuals (Peek and Blaser 2002). In this population, surface mucous cells are targeted by infection, but H. pylori are only rarely found in gland mucous cells in the deeper portion of the gastric mucosa (Hidaka et al. 2001; Matsuzawa et al. 2003), suggesting the presence of a defense mechanism in these cells.

Indeed, gland mucous cells secrete mucins containing α1,4-N-acetylglucosamine (α4GlcNAc)-capped core 2-branched O-glycans (Ishihara et al. 1996), and our recent studies indicate that α4GlcNAc-capped core 2-branched O-glycans expressed on soluble CD43, prepared by utilizing α1,4-N-acetylglucosaminyltransferase (α4GnT) cDNA (Nakayama et al. 1999; Zhang et al. 2001), inhibit H. pylori growth in a dose-dependent manner (Kawakubo et al. 2004). We also showed that gland mucins containing α4GlcNAc-capped O-glycans isolated from human gastric mucosa, such as MUC6 (Zhang et al. 2001; Nordman et al. 2002), inhibit H. pylori growth (Kawakubo et al. 2004). By contrast, surface mucins lacking α4GlcNAc-capped core 2-branched O-glycans, such as MUC5AC (Zhang et al. 2001; Nordman et al. 2002), facilitate H. pylori growth.
suggesting the presence of factors stimulating *H. pylori* growth in the surface mucins (Kawakubo et al. 2004). Growth inhibition by α4GlcNAc-capped core 2-branched O-glycans is apparently due to inhibition of cholesterol α-glucosyltransferase (HP0421), which synthesizes α-glucosyl cholesterol (Kawakubo et al. 2004; Fukuda et al. 2006). α-Glucosyl cholesterol and its derivatives are major components of the cell walls of *Helicobacter* species and constitute more than 25% of total cell wall lipids of *H. pylori*, reaching almost 130 µM (Hirai et al. 1995; Haque et al. 1996). In addition, it has been reported recently that α-glucosyl cholesterol abrogates phagocytosis of *H. pylori* and compromises subsequent T cell activation directed toward *H. pylori* (Wunder et al. 2006). Conversely, the increased amount of cholesterol resulted in increased phagocytosis of *H. pylori* and increased T cell responses toward *H. pylori*. These results demonstrate that α-glucosylation of cholesterol in *H. pylori* facilitates both infectivity and pathogenicity of *H. pylori*.

We recently cloned genomic DNA encoding HP0421 from *H. pylori* and related *Helicobacter* species and expressed it in *Escherichia coli* (Lee et al. 2006). We found that HP0421 activity was inhibited by core 2-branched mucin-type O-glycans containing α1,4GlcNAc residues (α4GlcNAc-core 2) (Lee et al. 2006). However, it is unclear how HP0421 acts on substrates and whether oligosaccharides inhibit *H. pylori* growth (Lebrun et al. 2006; Lee et al. 2006). Here, we first measured kinetics of HP0421 activity on acceptor and donor substrates. We found that UDP displays competitive inhibition toward HP0421 when the concentration of uridine diphosphogluco (UDP-Glc) is varied. When we analyzed the effect of chemically synthesized α-glucosyl cholesterol on HP0421, we found that it displays mixed-type inhibition. We also showed that α1,4GlcNAc-capped core 2-branched O-glycan exhibits a competitive inhibition toward HP0421. Finally, we showed that α4GlcNAc-capped core 2-branched O-glycan efficiently inhibits *H. pylori* growth, while core 2-branched O-glycan devoid of an α4GlcNAc-capping structure exhibits no inhibitory effect. These results combined indicate that derivatives of α4GlcNAc-capping core 2 O-glycan may be useful to treat patients infected with *H. pylori*.

### Results

#### Synthesis of α-glucosyl cholesterol by HP0421

We previously cloned genomic DNA encoding HP0421 from *H. pylori* and other *Helicobacter* species and produced soluble His-tagged HP0421 (Lee et al. 2006). After the expression in *E. coli*, the enzyme was isolated from solubilized *E. coli* periplasm on a Ni-NTA column and dialyzed against a Hepes-NaOH buffer (pH 7.5) to remove imidazole. This preparation resulted in a single protein band of an apparent molecular weight of ~39 kDa based on SDS–gel electrophoresis (Lee et al. 2006). Since the calculated molecular weight is 46,030 Da, this protein has an anomalous electrophoretic mobility. Because cholesterol is barely soluble without detergent, 0.1% Triton X-100 was added to the incubation mixture.

#### Inhibition of HP0421 activity by UDP and cholesterol

To determine how HP0421 acts on acceptor and donor substrates, enzyme activity was measured at various UDP-Glc concentrations, while fixed amounts of UDP or cholesterol were added as inhibitors. The Lineweaver–Burk plot of the inhibition profile of UDP in the presence of UDP-Glc intersected at the Y-axis, indicating competitive inhibition (Figure 1A). In contrast, cholesterol did not exhibit competitive inhibition in the presence of UDP-Glc and showed mixed-type inhibition (Figure 1B). These results suggest that cholesterol is likely...
Inhibition of HP0421 by α4GlcNAc-capped oligosaccharide

Previously, we demonstrated that HP0421 can be inhibited by mucin-type O-glycans (Lee et al. 2006). The oligosaccharides tested are α4GlcNAc-core 2 [GlcNAcα1→3Galβ1→4GlcNAcα1→6(Galβ1→3)GalNAcα1→octyl], sialylated core 2 [NeuAcα2→3Galβ1→4GlcNAcα1→6(Galβ1→3)GalNAcα1→octyl], and core 1 backbone [GlcNAcβ1→6(Galβ1→3)GalNAcα1→p-nitrophenol], and core 1 backbone (Galβ1→3GalNAcα1→p-nitrophenol) (Lee et al. 2006).

Among these mucin-type O-glycans, we found that α4GlcNAc-capped core 2-branched O-glycan, α4GlcNAc-core 2 was the best inhibitor. To determine how this oligosaccharide inhibits HP0421, HP0421 was incubated with varying UDP-Glc concentrations in the presence of 5 mM cholesterol, and then α4GlcNAc-core 2 oligosaccharide was added. Lineweaver–Burk analysis shows that α4GlcNAc-core 2 oligosaccharide inhibits HP0421 in a competitive manner (Figure 7). Using the DNRPEASY program, we found that $K_i$ for α4GlcNAc-core 2 is 418 ± 60 µM while $K_i$ for UDP is 22.9 ± 1.1 µM. This value is almost identical to that obtained previously (Lee et al. 2006), whereas IC$_{50}$ is 0.75 mM for monosialylated and disialylated core 2 O-glycan (Lee et al. 2006).

Since the $K_i$ value for α4GlcNAc-core 2 is 0.42 mM and αGlcNAc-core 2 is a competitive inhibitor, we can deduce the relationship between $K_i$ and IC$_{50}$ as follows:

$$K_i = IC_{50}/(1 + S/K_m), \quad \text{i.e. } IC_{50} = K_i (1 + S/K_m)$$

$$IC_{50} = 420 \mu M (1 + 3.6 \mu M/64 \mu M) = 440 \mu M$$

$$= 0.44 \text{mM}$$

where

- 420 µM is the $K_i$ value calculated above
- 3.6 µM is the $S$ value for UDP-Glc used to get the IC$_{50}$ value (Lee et al. 2006)
- 64 µM is the $K_m$ value for UDP-Glc.

α4GlcNAc-core 2 O-glycan inhibits H. pylori growth

Previously, we reported that recombinant CD43 expressing α4GlcNAc residues inhibits H. pylori (Kawakubo et al. 2004). However, we had not determined whether oligosaccharides containing α4GlcNAc residues inhibit H. pylori growth. Other structural requirements were not known. We thus evaluated the effect of α4GlcNAc-core 2 O-glycan, sialylated core 2 O-glycan (disialo-core 2), monosaccharides, or α-galactosyl cholesterol on H. pylori growth. Monosaccharide N-acetylgalosamine had no effect on H. pylori growth, but higher concentrations of p-nitrophenyl α-N-acetylgalactosamine inhibited microbial growth (Figure 8B and C). H. pylori growth was significantly inhibited by 0.5 mM core 2 O-glycan containing an α4GlcNAc-capping structure (Figure 8E). Inhibition by α4GlcNAc-core 2 O-glycan was more robust than that by α-galactosyl cholesterol, the product of HP0421 (Figure 8F). We also tested sialylated core 2 O-glycan, since this O-glycan is the second best inhibitor for HP0421 in vitro among the oligosaccharides tested (Lee et al. 2006), and represents a major O-glycan in mucins (Capon et al. 1992; Lloyd et al. 1996). Interestingly, sialylated core
Fig. 2. Steps in the chemical synthesis of α-glucosyl cholesterol. α-Glucosyl cholesterol was synthesized in eight steps starting from glucose and cholesterol. Eight synthetic steps can be grouped into five steps (A–E). Each step is fully described in Materials and Methods. The final product, α-glucosyl cholesterol, is designated Compound 6. Ac, acetyl; SEt, thioethyl; TBS, t-butyldimethyl silyl; Ph-<, benzylidene acetal.

Fig. 3. Mass spectrometric analysis of the α-glucosyl cholesterol product. α-Glucosyl cholesterol was subjected to ESI mass spectrometric analysis. The major signals correspond to [M + NH₄]⁺ (m/z = 566.43), [M + Na]⁺ (m/z = 571.37), and [M + K]⁺ (m/z = 587.37).
2 O-glycan without an α4GlcNAc-capping structure had no inhibitory activity (Figure 8D). These combined results indicate that an α4GlcNAc-capped core 2-branched O-glycan most efficiently inhibits H. pylori growth, and an α1,4GlcNAc residue is essential for that inhibition (see also Discussion).

Incubation of mono- and oligosaccharides in the H. pylori culture medium did not modify the oligosaccharides

To determine whether monosaccharides and oligosaccharides are modified during the incubation with H. pylori, these saccharides were isolated from the medium incubated with or without H. pylori. Thin layer chromatography of the medium itself showed several molybdenum sulfuric-positive compounds derived from the medium. In addition, a spot migrated at the same position as a control (M) was detected and the migration of the spot was essentially identical to that obtained in the medium cultured with H. pylori (Figure 9). These results indicate that the monosaccharides and oligosaccharides were not modified after incubation with H. pylori.

Discussion

The present study demonstrates that HP0421 acts in an ordered Bi-Bi manner, as shown for many other glycosyltransferases (Qasba et al. 2005). If the enzyme acts in an unordered Ping-Pong manner, neither UDP nor cholesterol should competitively inhibit its activity. The present study also demonstrates that a product like α-glucosyl cholesterol does not competitively inhibit HP0421. By contrast, α4GlcNAc-core 2 O-glycan apparently inhibits HP0421 in a competitive manner. These results strongly suggest that cholesterol is added to the enzyme/substrate complex after a complex between...
the enzyme and UDP-Glc forms (Figure 6). By this mechanism,  
the enzyme/UDP-Glc complex presumably induces a confor-
mational change such that flexible loops form to accommodate  
more favorable binding to an acceptor substrate.  

HP0421 utilizes UDP-Glc and then produces α-linked  
glucose attached to cholesterol. The HP0421 and α-1,4-
galactosyltransferase from Neisseria meningitidis  
have a common property to contain α-linked sugar residue. Indeed α-
1,4-galactosyltransferase also acts in an ordered Bi-Bi kinetic  
mechanism (Ly et al. 2002). Moreover, crystal structural studies  
show that α-1,3-galactosyltransferase from N. meningitides  
displays a conformation change in the COOH-terminal region of  
the enzyme as UDP and C-1 of the galactose is cleaved and the  
enzyme is bound with UDP (Zhang et al. 2003). These findings  
suggest that HP0421 also likely displays a similar confor-
mational change during the enzymatic reaction.  

As shown previously, inflammation associated with H. pylori  
infection is marked by an appearance of HEV-like vessels in  
the gastric mucosa, which likely recruit lymphocytes and ag-
gravate the inflammatory response. However, HEV-like vessels  
disappear once H. pylori in the stomach is eradicated by an-
tibiotics (Kobayashi et al. 2004). Individuals free of H. pylori  
recover normal gastric mucosa morphology and show only resid-
ual lymphocyte recruitment. Using a transgenic mouse model in  
which gastrin overexpression is driven by the insulin promoter  

Fig. 6. Catalytic mechanisms of HP0421. The kinetic data are consistent with  
an ordered Bi-Bi reaction mechanism. UDP-Glc binds to HP0421 (E) prior to  
cholesterol binding, and α-glucosyl cholesterol (Glc–CHL) is released prior to  
UDP release from the enzyme–UDP complex. The arrows indicate the  
directions of reactions. $K_p$ represents a kinetic constant to form a product,  
Glc–CHL.

Fig. 7. Lineweaver–Burk plot of HP0421 activity in the presence of  
α4GlcNAc-core 2 O-glycan. HP0421 was incubated with various  
concentrations of UDP-Glc in the presence of 5 µM cholesterol. Different  
fixed concentrations of α4GlcNAc-core 2 O-glycan,  
GlcNAcα1→4Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα1→octyl  
(pw2-149) were added to the enzyme reaction mixture. The concentrations of  
α4GlcNAc-core 2 are shown at the right.

Fig. 8. Inhibition of H. pylori growth by synthetic oligosaccharides and monosaccharides. H. pylori was cultured for 5 days in Mueller–Hinton broth supplemented  
with 5.5% horse serum containing various amounts of synthetic oligosaccharides and monosaccharides. Bacterial growth was measured at OD 600 nm, and the  
absorbance for control experiments at time 0 was subtracted from absorbance at later time points. Oligosaccharide and monosaccharide concentrations are 1 mM  
(red), 0.75 mM (orange), 0.5 mM (blue), 0.25 mM (green), 0.125 mM (brown), and control (closed circle). Two millimolar GlcNAc was also added in B (magenta).  
Each experiment consisted of three independent assays and a total of three independent experiments were carried out. Representative results are shown. In these  
assays, the standard deviation was less than 18%. Oligosaccharides and monosaccharides were initially dissolved in DMSO, and the final DMSO concentration in  
the culture medium was 1%. The growth curve in the absence of DMSO is shown as a dotted line (A).
and monosaccharides (25 nmol each in DMSO) as a marker and control are shown in M. Recent studies showed that *H. pylori* to take up exogenous cholesterol (Kawakubo et al. 2004; Lee et al. 2006) and incorporate into which most likely forms micelle with other lipids, must be also incorporated into epithelial cells (Wunder et al. 2006). Cholesterol in the medium, corporates cholesterol from the cholesterol-rich lipid-raft of epithelial cells (Wunder et al. 2006). Cholesterol in the medium, which most likely forms micelle with other lipids, must be also incorporated into *H. pylori* although its efficiency is very low (Wunder et al. 2006). During its incorporation, most cholesterol is α-glucosylated by HP0421 (Wunder et al. 2006). Although HP0421 apparently does not have a transmembrane domain, the enzyme is loosely associated with the membrane (Lebrun et al. 2006; Lee et al. 2006). The catalytic domain of HP0421 should face the cytoplasm to utilize UDP-Glc present in the cytoplasm, as suggested previously (Lebrun et al. 2006). α4GlNAc-core 2 oligosaccharides have their hydrophobic N-acetyl groups, which may be associated with micelle containing cholesterol and thus incorporated together with cholesterol into *H. pylori*. Since sialylated O-glycans are highly hydrophilic, they are unlikely associated with lipid micelle, thus unable to incorporate into *H. pylori*. These findings are consistent with previous findings that Gaβ1→4GlNAC can be incorporated into cells only after making it hydrophobic by full acetylation (Sarkar et al. 1995), and GlNAC can be incorporated into cells by having hydrophobic benzyl aglycon (Kuan et al. 1989). Future studies will be of significance to determine if α4GlNAC-capped core 2 O-glycan is enriched into cholesterol-rich raft in stomach, thus allowing their efficient incorporation into *H. pylori*.

While *H. pylori* can be eradicated from most patients by amoxicillin and erythromycin plus acid-lowering drugs, in a minority of patients *H. pylori* is resistant to those treatments. Moreover, this treatment exerts an effect on intestinal bacterial flora that can lead to unwanted side effects such as diarrhea (Rauws et al. 1988). The discovery of HP0421 inhibitors will be important because of their function as antibiotics against *H. pylori*. Since HP0421 is present only in *Helicobacter* species (Lee et al. 2006), inhibition of this enzyme acts as a *Helicobacter*-specific antibiotic, therefore, with minimal side effects (Wang et al. 2000), the inflammation–carcinoma sequence in the stomach was facilitated by *H. felis* infection. Gene microarray analysis showed that the pathological sequence is characterized by initial upregulation of inflammation-related genes such as the chemokine receptor CXCR4 and later CD74 and CXCL-5 (Kobayashi et al. 2007). Thus specific genes are upregulated sequentially during the inflammation–carcinoma progression in the stomachs of mice infected by *H. felis*.

The present study demonstrates that α4GlNAC-capped core 2-branched O-glycan inhibits *H. pylori* growth much more efficiently than sialylated core 2 O-glycan. The minimum concentration of α4GlNAC-core 2 required for growth inhibition is 0.25 mM, similar to IC₅₀ (0.44 mM) for the inhibition of HP0421 activity, and thus most likely inhibits *H. pylori*-directed inflammation. *H. pylori* does not synthesize cholesterol and thus needs to take up exogenous cholesterol (Kawakubo et al. 2004; Wunder et al. 2006). Recent studies showed that *H. pylori* incorporates cholesterol from the cholesterol-rich lipid-raft of epithelial cells (Wunder et al. 2006). Cholesterol in the medium, which most likely forms micelle with other lipids, must be also incorporated into *H. pylori* though its efficiency is very low (Wunder et al. 2006). During its incorporation, most cholesterol is α-glucosylated by HP0421 (Wunder et al. 2006). Although HP0421 apparently does not have a transmembrane domain, the enzyme is loosely associated with the membrane (Lebrun et al. 2006; Lee et al. 2006). The catalytic domain of HP0421 should face the cytoplasm to utilize UDP-Glc present in the cytoplasm, as suggested previously (Lebrun et al. 2006). α4GlNAC-core 2 oligosaccharides have their hydrophobic N-acetyl groups, which may be associated with micelle containing cholesterol and thus incorporated together with cholesterol into *H. pylori*. Since sialylated O-glycans are highly hydrophilic, they are unlikely associated with lipid micelle, thus unable to incorporate into *H. pylori*. These findings are consistent with previous findings that Gaβ1→4GlNAC can be incorporated into cells only after making it hydrophobic by full acetylation (Sarkar et al. 1995), and GlNAC can be incorporated into cells by having hydrophobic benzyl aglycon (Kuan et al. 1989). Future studies will be of significance to determine if α4GlNAC-capped core 2 O-glycan is enriched into cholesterol-rich raft in stomach, thus allowing their efficient incorporation into *H. pylori*.

![Image](https://academic.oup.com/glycob/article-abstract/18/7/549/1988106/106)
effects. Future studies are important to develop α4GlcNAc-core 2 O-glycan-based drug for the treatment of *H. pylori* infection.

### Materials and methods

**Expression of HP0421**

Genomic DNA encoding HP0421 was cloned from *H. pylori* ATCC 700392, whose entire genome has been sequenced (Tomb et al. 1997), as described (Lee et al. 2006). Genomic DNA encoding HP0421, originally subcloned into pBluescript SK (−) was amplified by PCR using primers containing NdeI (5') and XhoI (3') sites. The PCR product was subcloned into NdeI and XhoI sites of pTKNd6XH, which harbors a 6X Histidine tag at the C-terminus (Kim et al. 2003). The pTKNd6XH vector was obtained through the courtesy of Dr. Kwan-Hwa Park, Department of Food Science and Biotechnology, Seoul National University, Korea. In this vector, HP0421 expression is driven by an amylotic enzyme promoter (Kim et al. 1992). *E. coli* was transformed with pTKHd6XH-HP0421 by nucleoporation and cultured in an LB medium.

Bacteria were precipitated by a brief centrifugation and suspended in 100 mM Tris–HCl (pH 7.5) containing 15% glycerol and 5 mM dithiothreitol (DTT). After treatment with 20 µg mL⁻¹ lysozyme at 20°C for 5 min, samples were sonicated briefly and centrifuged at 20,000 g for 10 min. Supernatants were applied to a Ni-NTA column (GE Healthcare, Little Chalfont, United Kingdom) and washed with a 50 mM Tris–HCl (pH 7.5) containing 0.3 M NaCl and 20 mM imidazole.

Soluble HP0421-6XH was eluted with the same buffer containing 15% glycerol and 5 mM DTT. The enzyme obtained was kept at 4°C.

**Enzyme assay**

The enzyme solution was incubated in a 50 mM Hepes–NaOH buffer (pH 7.5) containing 20 µM UDP-Glc (50,000 cpm), 5 µM cholesterol, and 0.1% Triton X-100. After 10–30 min of incubation, the reaction was stopped by the addition of 80 mM HCl (final concentration) and then 10 volumes of ethyl acetate. After brief centrifugation, radioactivity in the ethyl acetate (upper) and aqueous (lower) phases were separated. The aqueous phase was dried by evaporation, ethyl sulfide (1.05 eq) and BF₃·EtOH (eight glucose equivalents) at room temperature. After it was dried by evaporation, benzaldehyde dimethyl acetal (1.2 eq) in methanol at room temperature for deacetylation and then reacted with benzaldehyde dimethyl acetal (1.2 eq) in the presence of pyridine (4.0 eq) and CH₂Cl₂ at −20°C (yield 95% of Compound 4). After thioglycoside 4 was in situ activated with bromine, the product was conjugated to cholesterol (2.0 eq) in the presence of AgOTf (2.0 eq) sym-collidine (1.8 eq) in CH₂Cl₂ at 0°C (yield 75%). Although the product was a mixture of α- and β-linked compounds (1:1), isomers were separated by silica gel column chromatography (Compound 5).

Finally, TBS was removed by TBAF (2.0 eq) in tetrahydrofuran, and benzylidene groups were removed by 80% acetic acid treatment at 70°C (yield 86%) yielding the desired product (Compound 6). Product identification was confirmed by mass spectrometric and NMR analyses.

GlcNAc₁→4Galβ₁→4GlcNAcβ₁→6(Galβ₁→3)GalNAc α₁→octyl, α4GlcNAc-core 2 O-glycan was newly synthesized by a two-step glycosylation strategy as reported (Wang et al. 2007). Briefly, GlcNAc₁→4Gal was conjugated to GlcNAc₃, forming ethylthio-glycosylated GlcNAc₁→4Galβ₁→4GlcNAc, using a modification of the published procedure (Buskas and Konradsson 2000). This compound was conjugated in situ with Galβ₁→3GlcNAc→octyl, resulting in GlcNAc₁→4Galβ₁→4GlcNAcβ₁→6(Galβ₁→3)GalNAc₁→octyl. The obtained compound exhibited NMR and mass spectra consistent with the desired compound (Wang et al. 2007). Disialylated core 2 O-glycan was synthesized as described previously (Sengupta et al. 2003). N-Acetylglucosamine and p-nitrophenyl α-N-acetylglucosamine were purchased from Sigma.

To determine the effect of these oligosaccharides on HP0421 activity, oligosaccharides were dissolved in a minimal amount of dimethyl sulfoxide (DMSO) and added to the reaction mixture described above to assay HP0421. A reaction mixture containing only DMSO served as a control. The enzyme assay was carried out in triplicate.

**Bacterial growth assay**

*H. pylori* (ATCC 43504; American Type Culture Collection, Manassas, VA) was cultured as described with minor modifications (Kawakubo et al. 2004). Briefly, *H. pylori* was cultured in Brucella broth supplemented with 10% horse serum at 35°C in 15% CO₂, followed by a second culture with Mueller–Hinton broth (Eiken Chemical, Tokyo, Japan) supplemented with 5.5% horse serum at 35°C in 15% CO₂. Diluted bacteria (1 × 10⁷ cells mL⁻¹) were then cultured further in Mueller–Hinton broth supplemented with 5.5% horse serum containing various amounts of synthetic oligosaccharides and monosaccharides. Oligosaccharides and monosaccharides were dissolved in DMSO and added to the culture medium to a final concentration of 1% DMSO. Control culture was carried out in the presence of 1% DMSO. Growth of bacteria cultured at 35°C in 15% CO₂ was determined by measuring the OD 600 nm every day.

**TLC of monosaccharides and oligosaccharides**

Synthetic oligosaccharides and monosaccharides in DMSO were cultured in Brucella broth containing 10% fetal bovine serum for 5 days at 35°C in 12% CO₂ atmosphere with or without *H. pylori* (1 × 10⁷ cells mL⁻¹). Final concentration of oligosaccharides and monosaccharides was 1 mM or 2 mM.
Control culture was performed in the presence of 1% DMSO. After incubation, sugar compounds were extracted from the culture media by adding 1.5 volumes of ethanol at −20°C for 1 h. After centrifugation, the supernatant was diluted with 5 volumes of water and then applied to a high-capacity C18 column (Alltech, Deerfield, IL). The fraction eluted with methanol was dried in a Speed Vac concentrator and dissolved in 90% methanol for TLC. TLC was developed in CH2Cl2/methanol (2:1 for GlcNAc, 3:1 for GlcNAc α-PNP, or 5:1 for α-glucosyl cholesterol and control) and chloroform/methanol/water (3:3:1 for disialo-core 2 and α4GlcNAc-core 2). Separated saccharides were visualized by heating at 80°C for 30 min after dipping in the molybdate reagent. The molybdate reagent was prepared by first dissolving 7.5 g of ammonium molybdate in water and then 5 g of cerium sulfate tetrahydrate was added. After dissolving both compounds by adding 29 mL of sulfuric acid, the final volume was brought to 500 mL by slowly adding water.

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Conflict of interest statement

We declare no conflict of interest.

Abbreviations

α4GlcNAc-core 2 O-glycan, GlcNAcα1→4Galβ1→4GlcNAcβ 1→6(Galβ1→3)GlcNAcα1→octyl; DMSO, dimethyl sulfoxide; HEV, high endothelial venules; TLC, thin layer chromatography; UDC, uridine diphosphoglucose.

References


