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Helicobacter pylori SabA Adhesin in Persistent Infection and Chronic Inflammation

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Helicobacter pylori adherence in the human gastric mucosa involves specific bacterial adhesins and cognate host receptors. Here, we identify sialyl-dimeric-Lewis x glycosphingolipid as a receptor for H. pylori and show that H. pylori infection induced formation of sialyl-Lewis x antigens in gastric epithelium in humans and in a Rhesus monkey. The corresponding sialic acid–binding adhesin (SabA) was isolated with the "retagging" method, and the underlying sabA gene (JHP662/HP0725) was identified. The ability of many H. pylori strains to adhere to sialylated glycoconjugates expressed during chronic inflammation might thus contribute to virulence and the extraordinary chronicity of H. pylori infection.

Leb antigen–independent binding. Earlier studies identified nearly identical babA genes at different H. pylori chromosomal loci, each potentially encoding BabA. The babA2 gene encodes the complete adhesin, whereas babA1 is defective because sequences encoding the translational start and signal peptide are missing (4). Our experiments began with analyses of a babA2–knockout mutant derivative of the reference strain CCUG17875 (hereafter referred to as 17875). Unexpectedly, this 17875 babA2 mutant bound to gastric mucosa from an H. pylori–infected patient with gastritis. A 17875 derivative with both babA genes inactivated (babA1A2) was constructed (14). This babA1A2 mutant also adhered (Fig. 1, B and C), which showed that adherence was not due to recombination to link the silent babA1 gene with a functional translational start and signal sequence. Pretreatment with soluble Leb antigen (structures in table S1) resulted in >80% lower adherence by the 17875 parent strain (Figs. 1E and 3C) but did not affect adherence by its babA1A2 derivative (Figs. 1F and 3C).

In contrast to binding to infected gastric mucosa (Fig. 1, A to I), the babA1A2 mutant did not bind to healthy gastric mucosa from a person not infected with H. pylori (Fig. 1, J to M), whereas the 17875 parent strain bound avidly (Fig. 1, M versus L). These results implicated another adhesin that recognizes a receptor distinct from the Leb antigen and possibly associated with mucosal inflammation.

Adherence was also studied in tissue from a special transgenic mouse that produces Leb antigen in the gastric mucosa, the consequence of expression of a human-derived α1,3 fucosyltransferase (FT) (15). Strain 17875 and the babA1A2 mutant each adhered to Leb mouse gastric epithelium (Fig. 2A, ii and iii), whereas binding of each strain to the mucosa of nontransgenic (FVB/N) mice was poor and was limited to the luminal mucus. Thus, Leb mice express additional oligosaccharide chains (glycans), possibly fucosylated, but distinct from the Leb antigen that H. pylori could exploit as a receptor.

delix antigen–mediated binding. To search for another receptor, thin-layer chromatography (TLC)–separated glycosphingolipids (GLSs) were overlaid with H. pylori cells and monoclonal antibodies (mAbs), as appropriate. These tests (i) showed that the babA1A2 mutant bound acid GSLs (Fig. 2B, iv, lanes 2 and 4), (ii) confirmed that it did not bind Leb GSL (lane 9), (iii) showed that its binding was abrogated by desialylation (lanes 3 and 5), and (iv) revealed that it did not bind sialylated GSLs of nonhuman origin (lane 1) [table S1, numbers 2 to 5, in (16)] (indicating that sialylation per se is not sufficient for

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adherence). In addition, the binding pattern of the \textit{babA1A2} mutant matched that of the mAb against sLex (Fig. 2B, ii versus iv), except that sLex-mono-GSL was bound more weakly by the \textit{babA1A2} mutant than by the mAb (Fig. 2B, iv) and no binding to sialyl-Lewis a-GSL was detected (table S1). Thus, the \textit{babA1A2} mutant preferably binds sialylated gangliosides, possibly with multiple Lex (fucose-containing) motifs in the core chain (thus, slower migration on TLC).

The \textit{babA1A2}-mutant strain was next used to purify a high-affinity binding GSL from human adenocarcinoma tissue (14). The \textit{H. pylori}–binding GSL was identified by mass spectrometry and \textsuperscript{1}H nuclear magnetic resonance (NMR) as the sialyl-dimeric-Lewis x antigen, abbreviated as the sdiLex antigen (Fig. 2, B and C) (14).

Further tests with soluble glycoconjugates showed that the 17875-parent strain bound both sLex and Leb antigens, whereas its derivative bound only sLex (Fig. 3A). Pretreatment of the \textit{babA1A2} mutant with sLex conjugate reduced its in situ adherence by more than 90% (Figs. 11 and 1C) (14) but did not affect adherence of the 17875-parent strain (Figs. 1H and 3C). Similarly, pretreatment of tissue sections with an mAb that recognizes sdiLex reduced adherence by the \textit{babA1A2} mutant by 72% (Fig. 3E).

Titration experiments showed that the \textit{babA1A2} mutant exhibited high affinity for the sdiLex GSLs, with a level of detection of 1 pmol (Fig. 2B, iv) (table S1, number 8). At least 2000-fold more (2 nmol) of the shorter sialyl-(mono)-Lewis x GSL was needed for binding (table S1, number 7). In contrast, its affinity \((K_a)\) for soluble conjugates was similar for mono and dimeric forms of sLex, \(1 \times 10^9 \text{M}^{-1}\) and \(2 \times 10^9 \text{M}^{-1}\), respectively (Fig. 3B) (16). These patterns suggest that the sialylated binding sites are best presented at the termini of extended core chains containing multiple Lewis x motifs, such as GSLs in cell membranes. Such optimization of steric presentation would be less important for soluble receptors.

The Scatchard analyses (16) also estimated that 700 sLex-conjugate molecules were bound per \textit{babA1A2}-mutant bacterial cell (Fig. 3B), a number similar to that of Leb conjugates bound per cell of strain 17875.

**Clinical isolates.** A panel of 95 European clinical isolates was analyzed for sLex binding (14). Thirty-three of the 77 \textit{cagA}\textsuperscript{+} strains (43%) bound sLex, but only 11% (2 out of 18) of \textit{cagA}\textsuperscript{-} strains \((P < 0.000).

However, deletion of the \textit{cagPAI} from strain G27 did not affect sLex binding, as had also been seen in studies of \textit{cagA}\textsuperscript{-} and Leb-antigen binding. Out of the Swedish clinical isolates, 39% (35 out of 89) bound sLex, and the great majority of these sLex-binding isolates, 28 out of 35 (80%), also bound the Leb antigen. Fifteen of the 35 (43%) sLex-binding isolates also bound the related sialyl-Lewis a antigen (sLea) (table S1, number 6), whereas none of the remaining 54 sLex-nonbinding strains could bind to sLea. The clinical isolates SM65 and WU12 (the isolate from the infected patient in Fig. 1, A to M) illustrate such combinations of binding modes (Fig. 3A). Of the two strains with sequenced genomes, strain J99 (17) bound sLex, sLea, and Leb antigens, whereas strain 26695 (18) bound none of them (Fig. 3A).

**Binding to inflamed tissue.** Gastric tissue inflammation and malignant transformation each promote synthesis of sialylated glycoconjugates (19), which are rare in healthy human stomachs (20). Immunohistochemical analysis showed that the sialylated antigens were located to the apical surfaces of the surface epithelial cells (fig. S2). To study gastric mucosal sialylation in an \textit{H. pylori} context, gastric biopsies from 29 endoscopy patients were scored for binding by the \textit{babA1A2} mutant and by the 17875 strain in situ, and for several markers of inflammation (table S2A) (14). Substantial correlations were found between \textit{babA1A2}-mutant adherence and the following parameters: (i) levels of neutrophil (PMN) infiltration, 0.47 \((P < 0.011)\); (ii) lymphocyte/plasma cell infiltration, 0.46 \((P < 0.012)\; (iii) mAb staining for sLex in surface epithelial cells and in gastric pit regions, 0.52 \((P < 0.004)\); and (iv) histological gastritis score, 0.40 \((P < 0.034)\). In contrast, there was no significant correlation between \textit{babA1A2}-mutant binding in situ and \textit{H. pylori} density in biopsies from natural infection (0.14, \(P < 0.47)\), nor between any inflammatory parameter and in situ adherence of strain 17875 (Leb and sLex binding).
The series of 29 patient biopsies was then compared to a series of six biopsies of *H. pylori*–noninfected individuals, and a considerable difference was found to be due to lower adherence of the *babA1A2* mutant (P < 0.001) (table S2B), whereas strain 17875 showed no difference.

**Binding to infected tissue.** Biopsy material from a Rhesus monkey was used to directly test the view that *H. pylori* infection stimulates expression of sialylated epithelial glycosylation patterns that can then be exploited by *H. pylori* for adherence [monkey biopsies in (14)]. This monkey (21) had been cleared of its natural *H. pylori* infection, and gastritis declined to baseline. Gastric biopsies taken at 6 months post-eradication showed expression of sLex in the gastric gland region (Fig. 4A) and no expression in the surface epithelium (Fig. 4A). In situ adherence of the *babA1A2* mutant was limited to gastric glands and was closely matched to the sLex expression pattern (Fig. 4B). No specific adherence to the surface epithelium was seen (Fig. 4B).

At 6 months post-therapy, this gastritis-free animal had been experimentally infected with a cocktail of *H. pylori* strains, of which the J166 strain (a *cagA*-positive, Leb and sLex binding isolate) became predominant a few months later. This led to inflammation, infiltration by lymphocytes [Fig. 4C, bluish due to hematoxylineosine (H/E)-staining], and microscopic detection of *H. pylori* infection (Fig. 4, E and F) (21). The virulent *H. pylori* infection led to strong sLex-antigen expression in the surface epithelium (Fig. 4C) and maintained expression in the deeper gastric glands (Fig. 4C); thus, a bi-layered expression mode supported strong binding of the *babA1A2* mutant to both regions (Fig. 4D). Bacterial pretreatment with sLex conjugate eliminated surface epithelial adherence and reduced gastric gland adherence by 88%. Thus, persistent *H. pylori* infection up-regulates expression of sLex antigens, which *H. pylori* can exploit for adherence to the surface epithelium.

**Binding to Leb transgenic mouse gastric epithelium.** We analyzed gastric mucosa of Leb mice for sLex antigen-dependent *H. pylori* adherence in situ [AIS, in (14)]. Pretreatment with the sLex conjugate reduced binding by *babA1A2*-mutant bacteria by more than 90% (Figs. 2A, vi, and 3D) but did not affect binding by its 17875 parent (Figs. 2A, v, and 3D). In comparison, pretreatment with soluble Leb antigen decreased adherence by >80% of the 17875 strain (Leb and sLex binding), whereas binding by the *babA1A2* mutant was not affected. mAb tests demonstrated sLex antigen in the gastric surface epithelium and pits of Leb mice (Fig. 2A, iv).

That is, these mice are unusual in producing sialyl as well as Leb glycoconjugates (even without infection), which each serve as receptors for *H. pylori*.

A finding that *H. pylori* pretreatment with Leb conjugate blocked its adherence to Leb mouse tissue, whereas sLex pretreatment did not, had been interpreted as indicating that *H. pylori* adherence is mediated solely by Leb antigen (22). However, because strain 17875 and its *babA1A2* mutant bound similar levels of soluble sLex conjugate (Fig. 3A), soluble Leb seems to interfere sterically with interactions between sLex-specific *H. pylori* adhesins and sLex receptors in host tissue (see also Fig. 1, E versus F). Because an excess of soluble sLex did not affect *H. pylori* binding to Leb receptors [Figs. 1H and 3C (in humans) and Figs. 2A, v, and 3D (in Leb-mice), the steric hindrance is not reciprocal. Further tests showed that liquid phase binding is distinct. A 10-fold excess of soluble unlabeled Leb conjugate (3 μg) along with 300 ng of 125I-sLex conjugate did not affect strain 17875 binding to soluble sLex glycoconjugate.

Thus, soluble Leb conjugates interfere with sLex-mediated *H. pylori* binding specifically when the sLex moieties are constrained on surfaces. The lack of reciprocity in these Leb-sLex interference interactions contributes to our model of receptor positioning on cell surfaces.

**SabA identified.** We identified the sLex-binding adhesin by "retaging." This technique exploits a receptor-bound multifunctional biotinylated crosslinker, and ultraviolet (UV) irradiation to mediate transfer of the biotin tag to the bound adhesin (4). Here, we added the crosslinker to sLex conjugate and used more UV exposure (14) than had been used to isolate the BabA adhesin (4) to compensate for the lower affinity of the sLex than the Leb-specific adhesin for cognate soluble receptors (Fig. 3B). Strain J99 was used, and a 66-kDa protein was recovered (Fig. 5A). Four peptides identified by mass spectrometry (MS)–matched peptides encoded by gene JHP662 in strain J99 (17) (gene HP07025 in strain 26695) (18). Two of the four peptides also matched those from the related gene JHP659 (HP0722) (86% protein level similarity to JHP662) (fig. S1).

To critically test if JHP662 or JHP659 encodes SabA, we generated *camR* insertion alleles of each gene in strain J99. Using radiolabeled glycoconjugates, we found that both sLex...
and sLea antigen-binding activity was abolished in the JHP662 (sabA) mutant, but not in the JHP659 (sabB) mutant. Thus, the SabA adhesin is encoded by JHP662 (HP0725). This gene encodes a 651-aa protein (70 kDa) and belongs to the large hop family of H. pylori outer membrane protein genes, including babA (17, 18). The sabA gene was then identified by PCR in six sLeb-binding and six non-sLex-binding Swedish isolates, which suggests that sabA is present in the majority of H. pylori isolates (14).

Parallel studies indicated that the sabA inactivation did not affect adherence mediated by the BabA adhesin (Fig. 5B, i) and that pretreatment of the J99/sabA mutant with soluble Leb antigen prevented its binding to gastric epithelium (Fig. 5B, ii). This implies that the SabA and BabA adhesins are organized and expressed as independent units.

Nevertheless, Leb conjugate pretreatment of J99 (BabA+ and SabA+) might have interfered with sLex antigen–mediated adherence (see Fig. 1E). To determine if this was a steric effect of the bulky glycoconjugate on exposure of SabA adhesin, single babA and sabA mutant J99 derivatives were used to further analyze Leb and sLex adherence (Fig. 5C, i to iv). Both single mutants adhered to the inflamed gastric epithelial samples, whereas the babAsabA (double) mutant was unable to bind this same tissue.

**Instability of sLex binding.** When screening for SabA mutants, we also analyzed single colony isolates from cultures of parent strain J99 (which binds both sLex and Leb antigens) (Fig. 3A), which indicated that 1% of colonies had spontaneously lost the ability to bind sLex. Similar results were obtained with strain 17875 (Fig. 3A) with an OFF (non-sLex-binding) variant called 17875/Leb. In contrast, each of several hundred isolates tested retained Leb antigen–binding capacity.

Upstream and within the start of the sabA gene are poly T/CT tracts that should be hotspots for ON/OFF frameshift regulation (see HP0725 in (18)), which might underlie the observed instability of sLex-binding activity. Both strains’ genome sequences, 26695 and J99, demonstrate CT repeats that suggest sabA to be out of frame (six and nine CTs, respectively) (17, 18). Four sLex-binding and four non–sLex-binding Swedish isolates, and in addition strain J99, were analyzed by PCR for CT repeats, and differences in length were found between strains. Ten CT repeats [as compared to nine in (17)] were
found in the sLex-binding J99 strain, which puts this ORF in frame, and could thus explain the ON bindings. These results further support the possibility for a flexible locus that confers ON/OFF binding properties (23).

Dynamics of sialylation during health and disease. Our analyses of H. pylori adhesion provide insight into human responses to persistent infections, where gastritis and inflammation elicit appearance of sLex antigens and related sialylated carbohydrates in the stomach mucosa, which cag\(^+\) (virulent) H. pylori strains by adaptive mechanisms exploit as receptors in concert with the higher affinity binding to Leb. These two adhesion modes may each benefit H. pylori by improving access to nutrients leached from damaged host tissues, even while increasing the risk of bactericidal damage by these same host defenses (Fig. 6). In the endothelial lining, sialylated Lewis-glycans serve as receptors for selectin cell adhesion proteins that help guide leukocyte migration and thus regulate strength of response to infection or injury (24). For complementary attachment, the neutrophils themselves also express sialylated Lewis glycans, and such neutrophil glycans allow binding and infection by human granulocytic erlichiosis (25). However, sialylated glycoconjugates are low in healthy gastric mucosa but are expressed during gastritis. This sialylation was correlated with the capacity for SabA-dependent, but not BabA-dependent, H. pylori binding in situ. Our separate tests on Rhesus monkeys for experimental H. pylori infection confirmed that gastric epithelial sialylation is induced by H. pylori infection. In accord with this, high levels of sialylated glycoconjugates have been found in H. pylori–infected persons, which decreased after eradication of infection and resolution of gastritis (26). Thus, a sialylated carbohydrate used to signal infection and inflammation and to guide defense responses can be co-opted by H. pylori as a receptor for intimate adherence.

High levels of sialylated glycoconjugates are associated with severe gastric disease, including dysplasia and cancer (27, 28). Sialylated glycoconjugates were similarly abundant in parietal cell–deficient mice (22). sLex was also present in the gastric mucosa of transgenic Leb mice (Fig. 2A). Whether this reflects a previously unrecognized pathology stemming from the abnormal (for mice) gastric synthesis of \(\alpha1.3/\alpha1.5\)T or Leb antigen, or from fucosylation of already sialylated carbohydrates, is not known.

Persons with blood group O and “nonsecretor” phenotypes (lacking the ABO blood group–antigen synthesis in secretion such as saliva and milk) are relatively common (e.g., ~45 and ~15%, respectively, in Europe), and each group is at increased risk for peptic ulcer disease (29). The H1 and Leb antigens are abundant in the gastric mucosa of secretors (of blood group O) (6), but not in nonsecretors, where instead the sLex and sLea antigens are found (30). The blood group O–disease association was postulated to reflect the adherence of most cag\(^+\) H. pylori strains to H1 and Leb antigens (3). We now suggest that H. pylori adherence to sialylated glycoconjugates contributes similarly to the increased risk of peptic ulcer disease in nonsecretor individuals.

Adaptive and multistep-mediated attachment modes of H. pylori. Our findings that the SabA adhesin mediates binding to the structurally related sialyl-Lewis a antigen (sLea, in table S1) is noteworthy because sLea is an established tumor antigen (31) and marker of gastric dysplasia (27), which may further illustrate H. pylori capacity to exploit a full range of host responses to epithelial damage. The H. pylori BabA adhesin binds Leb antigen on glycoproteins (32), whereas its SabA adhesin binds sLex antigen in membrane glycolipids, which may protrude less from the cell surface. Thus, H. pylori adher-
ence during chronic infection might involve two separate receptor-ligand, interactions—one at “arm’s length” mediated by Leb, and another, more intimate, weaker, and sLex-mediated adherence. The weakness of the sLex-mediated responses are most vigorous (Fig. 6C). In summary, we found that H. pylori infection elicits gastric mucosal sialylation as part of the chronic inflammatory response and that many virulent strains can exploit Selectin mimicry and another mechanism of sialyl binding activity and inflammation, and a sum-

## References and Notes

14. Strains and culture, adherence in situ (AIS), and adhesins have been characterized by allowing escape from sites where bactericidal host defense responses are most vigorous (Fig. 6C). In summary, we found that H. pylori infection elicits gastric mucosal sialylation as part of the chronic inflammatory response and thus “home in” on inflammation-activated domains of sialylated epithelium, complementing the baseline level of Leb receptors. The spectrum of H. pylori adhesion–receptor interactions is complex and can be viewed as adaptive, contributing to the extraordinary chronicity of H. pylori infection in billions of people worldwide, despite human genetic diversity and host defenses.

### Supporting Online Material

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### References and Notes

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**Reports**

## An Aligned Stream of Low-Metallicity Clusters in the Halo of the Milky Way

**Suk-Jin Yoon** and Young-Wook Lee

One of the long-standing problems in modern astronomy is the curious division of Galactic globular clusters, the “Oosterhoff dichotomy,” according to the properties of their RR Lyrae stars. Here, we find that most of the lowest metallicity ([Fe/H] < −2.0) clusters, which are essential to an understanding of this phenomenon, display a planar alignment in the outer halo. This alignment, combined with evidence from kinematics and stellar population, indicates a captured origin from a satellite galaxy. We show that, together with the horizontal-branch evolutionary effect, the factor producing the dichotomy could be a small time gap between the cluster-formation epochs in the Milky Way and the satellite. The results oppose the traditional view that the metal-poorest clusters represent the indigenous and oldest population of the Galaxy.

More than 60 years ago, Oosterhoff (1) discovered that Galactic globular clusters could be divided into two distinct groups according to the period of type ab RR Lyrae variables (P_m). This dichotomy was one of the earliest indications of systematic difference among globular clusters, whose reality has been strengthened by subsequent investigations (2). Given that most characteristics of Galactic globular clusters appear to be distributed in a continuous way, it is unusual that a quantity used to characterize variable stars falls into two rather well-defined classes. Moreover, the two groups are known to differ in metal abundance (3) and kinematic properties (4), which may indicate distinct origins. Whatever the reasons for the dichotomy, the question of whether the two groups originated under fundamentally different conditions is of considerable interest regarding the formation scenarios of the Galactic halo. Despite many efforts during the last decades, the origin of this phenomenon still lacks a convincing explanation.

Figure 1 shows the Oosterhoff dichotomy. Clusters belong to groups I and II if their values of (P_m) fall near 0.55 and 0.65 days, respectively (5). Group I is more metal-rich than group II (3). Based on our horizontal-branch (HB) population models (6, 7), we have found that the presence of the relatively metal-rich (−1.9 < [Fe/H] < −1.6) clusters in group II (hereafter group II-a) can be understood by the sudden increase in (P_m) at [Fe/H] ~ −1.6 (indicated by a blue line in Fig. 1). As [Fe/H] decreases, HB stars get hotter (i.e., the HB morphology gets bluer), and there is a certain point at which the zero-age portion of the HB just crosses the...