SabA Is the *H. pylori* Hemagglutinin and Is Polymorphic in Binding to Sialylated Glycans

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Adherence of *Helicobacter pylori* to inflamed gastric mucosa is dependent on the sialic acid–binding adhesin (SabA) and cognate sialylated/fucosylated glycans on the host cell surface. By in situ hybridization, *H. pylori* bacteria were observed in close association with erythrocytes in capillaries and post-capillary venules of the lamina propria of gastric mucosa in both infected humans and Rhesus monkeys. In vivo adherence of *H. pylori* to erythrocytes may require molecular mechanisms similar to the sialic acid–dependent in vitro agglutination of erythrocytes (i.e., sialic acid–dependent hemagglutination). In this context, the SabA adhesin was identified as the sialic acid–dependent hemagglutinin based on sialidase-sensitive hemagglutination, binding assays with sialylated glycoconjugates, and analysis of a series of isogenic *sabA* deletion mutants. The topographic presentation of binding sites for SabA on the erythrocyte membrane was mapped to gangliosides with extended core chains. However, receptor mapping revealed that the NeuAcα2–3Gal-disaccharide constitutes the minimal sialylated binding epitope required for SabA binding. Furthermore, clinical isolates demonstrated polymorphism in sialyl binding and complementation analysis of *sabA* mutants demonstrated that polymorphism in sialyl binding is an inherent property of the SabA protein itself. Gastric inflammation is associated with periodic changes in the composition of mucosal sialylation patterns. We suggest that dynamic adaptation in sialyl-binding properties during persistent infection specializes *H. pylori* both for individual variation in mucosal glycosylation and tropism for local areas of inflamed and/or dysplastic tissue.

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Introduction

The gastric pathogen *Helicobacter pylori* exhibits specific tropism for gastric mucosa in human populations worldwide [1]. Adherence to gastric epithelium may benefit the bacterium by placing it in close contact with epithelial surfaces and nutrients leaching from host cells that are damaged by local inflammation processes. The size of the *H. pylori* genome is only one-third of that of *Escherichia coli*, with ensuing limitations in metabolic pathways [2] and adoption of an adhesive and intracellular parasitic lifestyle. In addition, binding to highly glycosylated mucins in the mucus layer closest to the epithelium may stabilize *H. pylori* colonization and thus avoid clearance of infection caused by high epithelial turnover and shedding of the mucus layer [3]. *H. pylori* has been shown to adhere to erythrocytes and neutrophils in vitro [4,5], and virulence-associated cag^{+} *H. pylori* strains have been shown to invade both the gastric mucosa and individual cells [6–10]. Thus, the ability to adhere may also affect the outcome of *H. pylori* infection by facilitating focused delivery of effector molecules into the host cell [11,12]. Consequently, during infection, tissue invasion and migration of *H. pylori* bacterial cells through the endothelial lining of capillaries and post-capillary venules followed by adherence to blood cells may result in transfer and systemic dissemination of *H. pylori*.

H. pylori adapts to the gastric environment by binding to oligosaccharides (glycans) of various complexities, so-called receptors or binding epitopes for establishment of infection in different parts of the mucosa. These glycans are presented on cell surfaces by glycoproteins and glycosphingolipids, and in the gastric mucus by MUC5AC and MUC6 mucin molecules

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Abbreviations: BabA, blood group antigen–binding adhesin; BSA, bovine serum albumin; HpaA, *H. pylori* adhesin A; Leb, Lewis b; SabA, sialic acid–binding adhesin; sdiLex, sialyl-dimeric Lewis x; sia-HA, sialic acid–dependent hemagglutination; sLex, sialyl-Lex; sLn, sialyl-lactosamine

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Synopsis

Helicobacter pylori infections are very common worldwide and cause chronic inflammation in the stomach (gastritis), which may progress to peptic ulcer disease and stomach cancer. In the gastric epithelium, *H. pylori* infections induce expression of inflammation-associated "sialylated" carbohydrates. The ability to bind to the glycosylated epithelial cells is considered to be essential for *H. pylori* to cause persistent infection and disease. Here the authors show that during established infection, *H. pylori* also binds to red blood cells in gastric mucosal blood vessels in both infected humans and Rhesus monkeys. The authors found that "sialic acid–binding adhesin" (SabA), is the bacterial surface protein that mediates binding of *H. pylori* to red blood cells. Furthermore, they show that clinical *H. pylori* isolates demonstrate "polymorphism" in their abilities to bind various sialylated carbohydrates, and that the variation in binding properties depends on the sialic acid–binding adhesin protein itself. This variability may adapt the binding properties of *H. pylori* both to individual hosts and the changing epithelial glycosylation patterns during chronic inflammation. Continuous adaptation to inflamed tissue during persistent infections is probably a general feature of microbial pathogens, although their binding properties have not yet been explored in detail.

[13]. The *H. pylori* glycan receptors include fucosylated ABO blood group antigens [14], glycans with charged modifications such as sialic acid [15] and sulfate [16], and, in addition, unsubstituted core chain glycans [17]. The many different receptor structures described for mucosal adherence suggest that, similar to multiadhesive pathogens such as *Pseudomonas aeruginosa, H. pylori* expresses a range of different attachment proteins, so-called adhesins [18]. The best-characterized interaction between *H. pylori* adhesins and host cell receptors is that between the blood group antigen–binding adhesin (BabA) and the ABO blood group and Lewis b (Leb) antigens [14]. ABO/Leb blood group antigens are best known from erythrocytes, but they are also highly expressed in the epithelium and mucus lining in the oro-gastrointestinal tract [19], where they are known as histo–blood group antigens [20].

As shown in a previous study, a *H. pylori babA* deletion mutant that cannot bind ABO/Leb blood group antigens nevertheless binds to gastric epithelium [15]. Further analysis showed that the *babA* mutant preferentially bound to inflamed gastric mucosa, and that binding was mediated by sialylated glycans such as sialyl-Lewis x (sLex) and sialyl-Lewis a (sLea). The sLex and sLea glycans are better known as the glycan binding sites for the selectin family of cell adhesion molecules. Expression of selectin molecules is activated by inflammatory responses, and they have important roles in recruitment of white blood cells from circulation to the tissue in need (reviewed in [21]). Infection of the gastric mucosa by *H. pylori* results in inflammatory responses, with concomitant expression of sialylated glycans. *H. pylori* has been suggested to exploit mechanisms of "selectin mimicry" to "home in" on inflamed gastric tissue by binding to epithelial sLex and sLea. By analysis of both gastric biopsy material from individuals with gastritis or peptic ulcer disease and experimentally infected Rhesus monkeys, strong correlations were found between expression of sialylated Lewis antigens, gastritis, and *H. pylori* infection. Similar to BabA, the sialic acid–binding adhesin, SabA, was purified and identified by the retagging technique based on its affinity for sLex [15].

Sialylated glycoconjugates are common binding sites for both Gram-negative and Gram-positive bacteria [22], influenza and adenoviruses [23,24], and parasites [25]. Hemagglutination analysis is an established method for characterization of microbial adherence to host cell surfaces. Analysis of sialic acid–dependent hemagglutination (sia-HA) has been facilitated by the easy removal of sialylated epitopes by sialidase enzyme, and has been refined in some cases by complementary enzymatic resialylation of cell surfaces [22,26]. Soon after its discovery, *H. pylori* was shown to agglutinate erythrocytes [4]. The activity was suggested to be dependent on sialic acid since the HA activity was lost by prior sialidase treatment of erythrocytes [27]. About one-third of fresh clinical *H. pylori* isolates demonstrate sialidase-sensitive HA [28]. This figure is similar to the prevalence of sLex binding among clinical isolates [15]. The sialic acid–binding epitope was characterized as NeuAcα2–3Gal since sialyl-lactose could competitively inhibit sia-HA [27]. The *N*-acetyl-neuraminyl-lactose–binding hemagglutinin was originally affinity purified by use of a sialylated serum protein and denoted *H. pylori* adhesin A (HpaA) [29]. However, results from later studies have questioned the role of HpaA in sia-HA; first, a *hpaA* deletion mutant demonstrated no reduction in sia-HA activity; [30] and second, immunogold localization analysis suggested that HpaA is most likely a flagellar sheath protein [31]. Similarly, the *H. pylori* neutrophil-activating protein (HP-NAP) has been described to exhibit sialic acid–binding properties [32]. However, a J99 HP-NAP depleted mutant was no different from the parent strain in sialic acid–binding or in sia-HA properties [33].

Here we report that *H. pylori* can be found on erythrocytes in capillaries and post-capillary venules in gastric mucosa of infected humans and Rhesus monkeys. These results extend our earlier findings that *H. pylori* is a facultative intracellular bacterium that can leach from the lumen of the stomach into epithelial cells and the lamina propria [9], indicating that the bacterium may disseminate into the circulation by way of gastric mucosal capillaries. Our results also demonstrate that the SabA adhesin is the sought-after sialyl-dependent hemagglutinin of *H. pylori*. The preferred binding sites for SabA on the erythrocyte cell surface were mapped to extended ganglioside glycans. We also found a high level of polymorphism in sialyl-binding properties among clinical isolates, which suggest functional adaptation of SabA both to individual and disease-related differences in mucosal sialylation patterns.

Results

Adherence of *H. pylori* to Erythrocytes in Capillaries and Post-Capillary Venules in Gastric Mucosa of Infected Humans and Rhesus Monkeys

Rhesus monkeys and humans have very similar gastric anatomy, histology, and mucosal glycosylation patterns, and they can be naturally infected by *H. pylori*. In addition, *H. pylori* infection is associated with mucosal inflammation, gastritis [6], and sialylated mucosal glycosylation pattern [15]. Biopsies harvested from the gastric mucosa of humans and experimentally infected Rhesus monkeys were analyzed for spatial localization of *H. pylori* cells. Genta-stained and toluidine blue–stained sections of gastric mucosa (Figure 1A and 1B, respectively) revealed the presence of a few *H. pylori*

bacterial cells tightly associated with erythrocytes within capillaries and post-capillary venules in addition to the previously reported distribution of the bacterial cells in the lumen and foveolar epithelium [9]. To test if these bacterial cells were *H. pylori* that had invaded the gastric tissue of Rhesus monkeys and entered the microcirculation, in situ hybridization was performed. Thus, bacterial cells attached to the erythrocyte surfaces were identified by use of probes specific for *H. pylori* 16S RNA (Figure 1C). Next, the possibility that *H. pylori* can also invade blood vessels and adhere to erythrocytes in humans was analyzed. In situ hybridization in biopsies of infected human gastric mucosa revealed a similar localization of *H. pylori* bacterial cells (Figure 1D). These results suggest that *H. pylori* can reach the gastric mucosa capillaries, attach to erythrocytes, and perhaps disseminate throughout the body of both humans and Rhesus monkeys. Importantly, the number of bacterial cells in the endothelial lining was drastically lower compared to the foveolar epithelium (illustrated in Figure 1A). This difference in *H. pylori* bacterial cell density is consistent with the absence of clinical cases with overt sepsis caused by *H. pylori* infection.

Correlation between sia-HA and sLex Binding

A series of 99 Swedish clinical *H. pylori* isolates were tested for both sia-HA and for binding to sLex glycoconjugate. Sia-HA was assessed by desialylation of human erythrocytes with sialidase from *Clostridium perfringens,* a glycosidase that hydrolyses α2–3–, α2–6–, and α2–8–linked sialic acid in oligosaccharide chains of natural glycoconjugates, such as glycoproteins and glycosphingolipids. Enzymatic desialylation of the erythrocyte surfaces and subsequent removal of sialylated bacterial binding sites would result in reduced erythrocyte aggregation, and thus reduced HA titers. For the series of clinical isolates, sialidase-dependent shifts in HA titers ranged from -2 to ≥ 2, where positive values relate to the expected effect of sialidase on HA (i.e., the reduction in sialyl-dependent binding by one to three titer shifts). Negative values correspond to strains that, in contrast, increase HA titers due to removal of sialic acid (i.e., sialyl-independent HA). HA titers (1 to $\geq$ 2) were found for 27 isolates (27%), whereas 31 isolates (31%) showed increased HA titers (–1 to –2). The remaining 41 isolates (41%) displayed no change in sia-HA titers (Figure 2A).

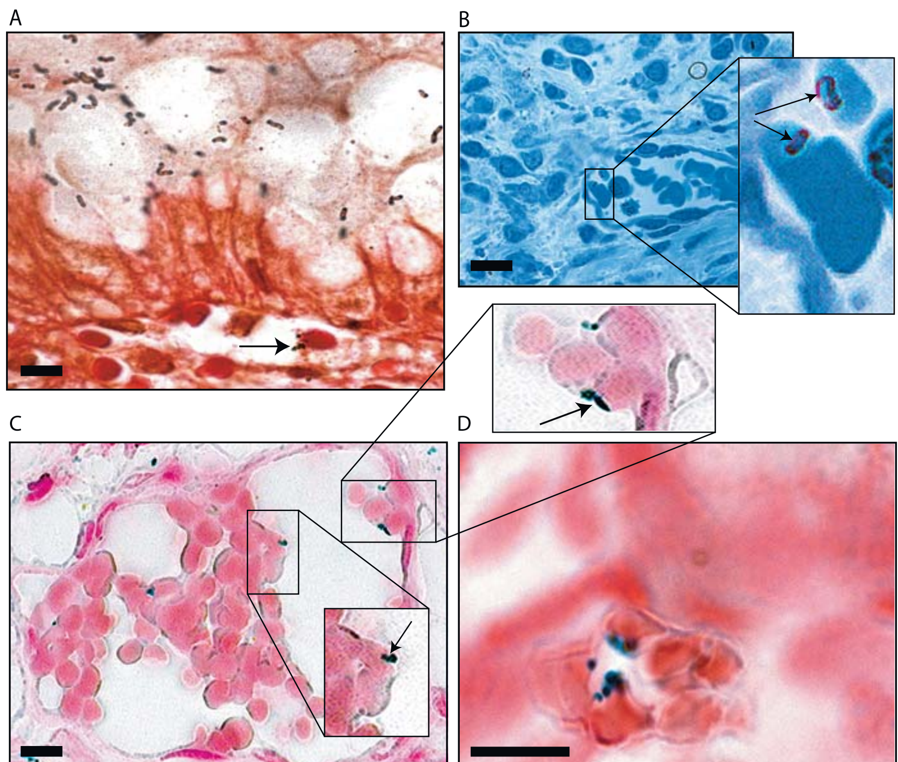

Figure 1. *H. pylori* Adheres to Erythrocytes in Capillaries and Post-Capillary Venules of Infected Humans and Rhesus Monkeys

(A) Genta-stained section of human gastric biopsy. Black spiral- and comma-shaped bacteria are observed in the lumen of the stomach, adherent to gastric epithelial cells, within the mucus globule of the cells. Bacterial cells (arrow) are also present in close contact to an erythrocyte within a capillary located in the supporting connective tissue of lamina propria of the mucosa.

(B) Section of human gastric biopsy stained with toluidine blue. A capillary vessel lined by endothelial cells is visible in the lamina propria of the mucosa. It contains several erythrocytes to which *H. pylori* are attached. Insert: higher magnification of two *H. pylori* (arrows) in close approximation to erythrocytes.

(C) Section of a Rhesus monkey gastric biopsy. In situ hybridization was performed using probes specific for *H. pylori* 16S rRNA, demonstrating the presence of several *H. pylori* apparently attached to erythrocyte surfaces of a post-capillary venule located in the lamina propria of submucosa. Inserts: higher magnification of *H. pylori* bacterial cells (arrows) in close approximation to erythrocytes.

(D) Section of a human gastric biopsy. In situ hybridization was performed using probes specific for *H. pylori* 16S rRNA. This high magnification of a capillary immediately adjacent to a gastric gland (on the top-right corner of the picture) demonstrates the presence of several *H. pylori* bacterial cells, stained blue, apparently attached to the surfaces of erythrocytes.

Bars = 5 μm.

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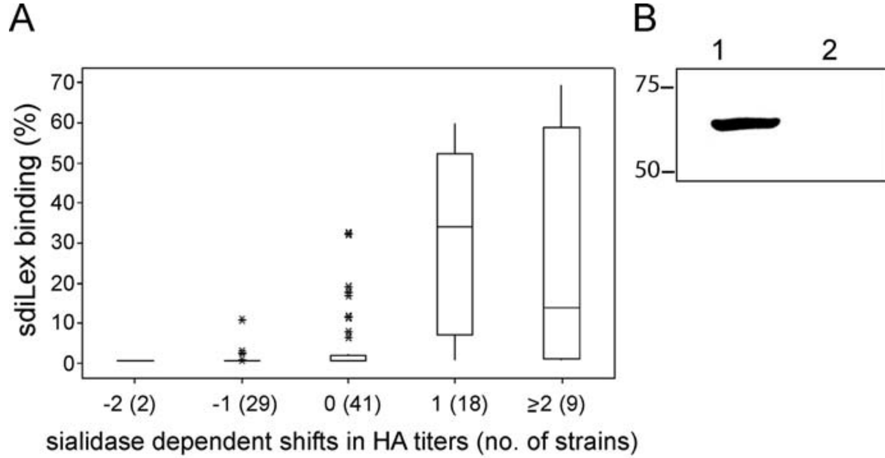

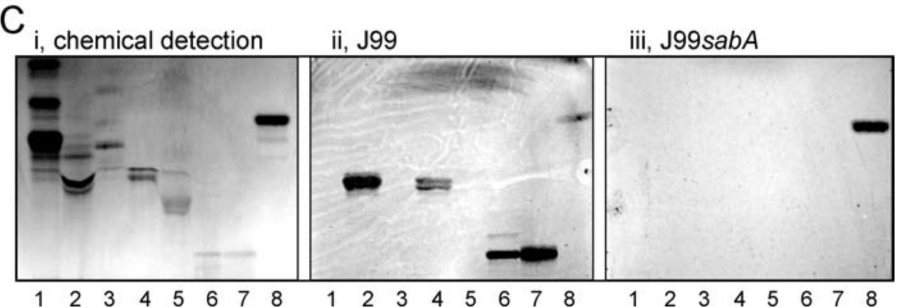

Figure 2. Characterization of Binding Properties of the SabA Adhesin, and Its siaHA Properties

(A) A panel of 99 Swedish clinical *H. pylori* isolates was tested for sia-HA properties and for binding to ^{125}I-sdiLex conjugate. The numbers on the *x* axis indicate the shifts in HA titers after sialidase treatment: positive values indicate lowered sia-HA titers (i.e., sia-HA, whereas negative values indicate increased HA titers (i.e., sialic acid–independent HA). No change in HA titer is indicated by 0. The *y* axis gives the percentage of bound sLex-conjugate.
(B) SabA was affinity-adsorbed to erythrocytes from a cell-surface protein extract of strain J99. Immunostaining using SabA antibodies confirmed the presence of SabA adsorbed onto the erythrocyte surfaces as a result of binding to sialylated glycans (lane 1), whereas SabA was completely absent when erythrocytes had been depleted of sialic acid by sialidase treatment prior to the test (lane 2). Molecular weight markers (in kDa) are indicated.
(C) Binding of *H. pylori* strains J99 and J99*sabA* to human erythrocyte glycosphingolipids. (i) Chemical detection by anisaldehyde. (ii–iii) Autoradiograms obtained by binding of ^{35}S-labeled *H. pylori* strain J99 and the J99*sabA* mutant, respectively, to separated glycosphingolipids. The lanes contain non-acid glycosphingolipids of human erythrocytes, 40 μg (lane 1); gangliosides of human erythrocytes, 40 μg (lane 2); GM3 ganglioside (NeuAcα2–3Galβ4Glcβ1Cer), 4 μg (lane 3); NeuAcα2–3-neolactotetraocylceramide (NeuAcα2–3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 μg (lane 4); NeuAcα2–6-neolactotetraocylceramide (NeuAcα2–6Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 μg (lane 5); G-10 ganglioside (NeuAcα2–3Galβ4GlcNAcβ6 (NeuAcα2–3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3-Galβ4Glcβ1Cer), 1 μg (lane 6); G9-B ganglioside (Galα3(Fucα2)Galβ4GlcNAcβ6 (NeuAcα2–3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 1 μg (lane 7); and reference gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer) of mouse feces, 4 μg (lane 8).
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For the series of 99 clinical isolates, HA titers were compared with prevalence of sLex binding. This was assessed by the use of ^{125}I-labeled sialyl-dimeric Lex (sdiLex) antigen conjugate. The sdiLex antigen consists of two repetitive and fucosylated Lex antigens terminally substituted with α2–3–linked sialic acid (Table 1). A total of 37 of 99 isolates (37%) could bind to sdiLex, and 22 of 37 (60%) of these isolates proficient in sdiLex binding also demonstrated reduction in sialyl-dependent binding (sia-HA properties). In comparison, among the 62 isolates that did not bind sdiLex, only five isolates (8%) demonstrated sia-HA properties. In the group of 31 isolates that instead increased their HA titers due to sialidase treatment, only one single isolate showed distinct sdiLex binding, two isolates bound weakly, and the great majority (28 isolates) completely lacked sdiLex-binding properties (Figure 2A).

A strong correlation was found between bacterial binding to sdiLex and HA titers resulting from sialidase treatment (correlation of rank = 0.547; $p < 0.001$) (Figure 2A). These results demonstrate that clinical isolates that are reduced in HA activity due to desialylation of erythrocytes by sialidase treatment constitute the group of *H. pylori* strains in which the great majority can bind sialylated Lewis antigens.

A *sabA* Deletion Mutant Identified SabA as the Sialyl-Dependent Hemagglutinin

Strain J99 has previously been shown to express the SabA adhesin and to bind sialylated Lewis glycans such as sdiLex, sLex, and sLea (Table 1). By comparison, the J99 *sabA* deletion mutant (J99*sabA*) has lost the ability to bind sialyl-Lewis antigens [15]. Here, the J99 and J99*sabA* strains were compared for sia-HA properties. The results showed that while strain J99 was positive for sia-HA, the J99*sabA* mutant is fully devoid of all sia-HA properties (Table 2, 16× [a] versus 0 [b]). This result is most consistent with SabA being the sialyl-dependent hemagglutinin of *H. pylori.* In comparison, the J99

Table 1. Soluble Glycoconjugates Used in This Study

Oligosaccharides	Structures
Leb	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4(Glc)-APD-HSA
sdiLex	NeuAcα2–3Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4(Glc)-APD-HSA
sLex	NeuAcα2–3Galβ4(Fucα3)GlcNAcβ3Galβ4(Glc)-APD-HSA
sLea	NeuAcα2–3Galβ3(Fucα4)GlcNAcβ3Galβ4(Glc)-APD-HSA
3′-sialyl-lactose	NeuAcα2–3Galβ4(Glc)-APD-HSA
6′-sialyl-lactose	NeuAcα2–6Galβ4(Glc)-APD-HSA
sLn(3)	NeuAcα2–3Galβ4GlcNAc-3 atom spacer-BSA
sLn(14)	NeuAcα2–3Galβ4GlcNAc-14 atom spacer-BSA

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babA deletion mutant (J99*babA*) was not affected in sia-HA, but instead behaved most similarly to the J99 parent strain (Table 2, 16x [c] versus [a]). Taken together, the results also demonstrate that binding by BabA to fucosylated blood group antigens does not confer erythrocyte aggregation and HA.

SabA Is Adsorbed to Sialylated Erythrocyte Surfaces

Binding of solubilized SabA protein was analyzed by affinity adsorption to sialylated erythrocyte surfaces. Here, a cell-surface protein extract from strain J99 was mixed with naive or sialidase-treated erythrocytes. Immunoblot analysis with antibodies against SabA showed that SabA was affinity absorbed onto intact and sialylated erythrocytes, whereas no SabA bound to sialidase-treated and sialic acid–depleted erythrocytes (Figure 2B).

Topographic Mapping of the Sialylated Binding Sites on Erythrocytes that Confer sia-HA by *H. pylori*

Sialylated erythrocyte antigens have been described in glycoproteins such as glycophorin A [22], in glycosphingolipids (gangliosides), and in polyglycosylceramides [34]. Sialylated bacterial binding sites were topographically localized on erythrocytes by functional discrimination between sialylated glycoproteins, which reach above the membrane level, and tight membrane–associated gangliosides. Erythrocytes were first treated with protease (trypsin) to destroy most glycoproteins at the erythrocyte surface, followed by treatment with sialidase to test for sialic acid–dependent binding sites. Proteolytic removal of cell-surface glycoproteins resulted in a distinct increase in sia-HA for both the J99 wt strain and the J99 *babA* mutant (Table 2, [d] and [e]). In contrast, S-fimbriated *E. coli,* which is known to hemagglutinate by binding to the sialylated erythrocyte glycoprotein glycophorin A (Table 2, [1]) [22], was most sensitive to protease treatment of erythrocytes, which even at low concentrations completely abrogated sia-HA (Table 2, [f] versus [g]). Similar to *H. pylori,* protease treatment of erythrocytes conferred stronger HA of P-fimbriated *E. coli,* which binds Galα4Gal antigens that are only present in glycolipids. This is most likely due to increased accessibility of the adhesive P-fimbriae for the Galα4Gal receptor epitopes present in membrane-close glycolipids (Table 2, [h] versus [i]). In addition, sialidase treatment and removal of the charged sialic acid residues could confer better exposure of the Galα4Gal receptor epitopes in glycan core chains and increased sia-HA (Table 2, [j] and [k] versus [h]). Taken together, the results show that the main part of sia-HA is conferred by sialyl-dependent binding of SabA to the tight membrane–associated and sialylated glycosphingolipids (i.e., gangliosides).

Table 2. Bacterial HA of Enzyme-Treated Erythrocytes

RBC Treatment	Dilution of Bacterial Cells				
	J99	J99*sabA*	J99*babA*	*E. coli* P-fim	*E. coli* S-fim
0 mg Tryp + 0 U Sia	16× (a)	– (b)	16× (c)	2× (h)	4× (f)
0 mg Tryp + 0.1 U Sia	–	–	–	8× (j)	– (l)
0.05 mg Tryp + 0U Sia	32× (d)	–	32× (e)	8× (i)	– (g)
0.05 mg Tryp+ 0.1U Sia	–	–	–	16× (k)	–

H. pylori strain J99 and the J99*sabA* and J99*babA* mutants were examined for HA of erythrocytes treated with different concentrations of trypsin (Tryp) and/or sialidase (Sia). As controls, P-fimbriated (P-fim) and S-fimbriated (S-fim) *E. coli* strains were analyzed for HA properties. The "Dilution of Bacterial Cells" (2x–32x) refers to the maximum bacterial dilution in which HA could be detected, and (–) indicates lack of HA. The letters (a–l) within parentheses are explained in the Results section "Topographic Mapping of the Sialylated Binding Sites on Erythrocytes that Confer sia-HA by *H. pylori*."
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SabA Binds to Gangliosides from Human Erythrocytes

The major ganglioside structures found in human erythrocytes are the GM3 ganglioside and NeuAcα2–3-neolactotetraosylceramide (Table 3; NeuAcα3SPG). In addition, a number of minor complex gangliosides have been described [35]. Binding of strain J99 to non-acid and sialylated glycosphingolipid fractions of human erythrocytes immobilized on thin-layer chromatography plates (i.e., solid phase presentation) was analyzed (Figure 2Cii, lanes 1 and 2, respectively). The NeuAcα2–3-neolactotetraosylceramide region of the acid fraction (lane 2) was positive for binding, while no binding by strain J99 to the non-acid glycosphingolipids was observed. A higher sensitivity was obtained when purified human erythrocyte gangliosides was used (lanes 3–7). As for the acid glycosphingolipid fraction (lane 2), strain J99 bound to purified NeuAcα2–3-neolactotetraosylceramide (lane 4). In addition, the bacteria bound the purified complex gangliosides G-10 and G9-B (lanes 6 and 7). These structures are minor components of the erythrocyte gangliosides, which may explain their lack of binding in the crude fraction (lane 2). Strain J99 did not bind the shorter GM3 ganglioside (lane 3), nor did it bind the α2–6–linked NeuAcα2–6-neolactotetraosylceramide (lane 5 and Table 3; NeuAcα6SPG). Interestingly, NeuAcα2–8 modifications to the NeuAcα2–6–

Table 3. Results from Binding of *H. pylori* to Human Erythrocyte Gangliosides

Gangliosides		Trivial Names	Structures	Binding
Monosialo-gangliosides	G1	NeuAc-GM3	NeuAcα2–3Galβ4Glcβ1Cer	–
	G2	NeuAcα3SPG	NeuAcα2–3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+
	G4	NeuAcα6SPG	NeuAcα2–6Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–
	G6	NeuAcα3-nLc$_6$	NeuAcα2–3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+++
	G9-B		Galα3(Fucα2)Galβ4GlcNAcβ6(NeuAcα2–3Galβ4Glc-NAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+++
Di-sialo-gangliosides		GD3	NeuAcα2–8NeuAcα2–3Galβ4Glcβ1Cer	–
	DG3	DPG	NeuAcα2–8NeuAcα2–3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–
	G-10/DG6		NeuAcα2–3Galβ4GlcNAcβ6(NeuAcα2–3Galβ4Glc-NAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+++
		GD1a	NeuAcα2–3Galβ3GalNAcβ4(NeuAcα2–3)Galβ4Glcβ1Cer	–
		GD1b	Galβ3GalNAcβ4(NeuAcα2–8NeuAcα2–3)Galβ4Glcβ1Cer	–

Binding of strain J99 is defined as follows: +++ denotes binding when less than 0.5 μg of the glycosphingolipid was applied on the thin-layer chromatogram, while + denotes occasional binding at 0.5 μg, and – denotes no binding even at 4 μg.
The glycosphingolipid nomenclature follows the recommendations by the International Union of Pure and Applied Chemistry–International Union of Biochemistry (IUPAC-IUB) Commission on Biochemical Nomenclature.
It is assumed that Gal, Glc, GlcNAc, GalNAc, and NeuAc are of the D-configuration, Fuc is of the L-configuration, and that all sugars are present in the pyranose form.
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substituted GD3 and DPG glycans seemingly interfered with SabA-mediated binding. Similarly, the GD1a glycan, which is similar to the NeuAcα2–3-neolactotetraosylceramide (NeuAcα3SPG), but with an additional NeuAcα2–3 residue linked to the core, had lost its binding epitope for SabA (Table 3), which further points to the importance of the steric presentation of the NeuAcα2–3Gal epitope for the SabA-mediated binding mode. The absolute requirement for SabA was demonstrated by the *sabA* deletion mutant strain J99*sabA*, which did not bind to any of the gangliosides tested (Figure 2Ciii), although there was consistent SabA-independent binding to the reference non-acid gangliotriaosylceramide (lane 8; [36]).

Characterization of *H. pylori* Binding Specificities to Sialylated Glycans

The sialylated receptor epitope for SabA was further characterized by use of structurally defined sialylated glycoconjugates of various complexities in terms of fucosylation and core chain and spacer unit lengths (Figure 3). The extended sdiLex antigen was recently shown to be the best receptor for SabA, which further suggests that fucosylation, sialylation, and length of the core chain are parameters that together form a high-affinity binding epitope for SabA. NeuAcα2–3-lactose and NeuAcα2–6-lactose were first compared for bacterial binding. Both structures are based on purified sialyl-lactose structures, but the glucose ring has been opened by reductive amination and used for conjugation to albumin, which leaves only the NeuAc-Gal disaccharide intact and available for bacterial binding. Strains J99, CCUG17875 (17875), 17875*babA1A2*, CCUG17874 (17874), SMI65, and SMI27 all bound the NeuAcα2–3-lactose conjugate, while none of the strains tested bound the NeuAcα2–6-lactose conjugate. The results suggest that presentation of the sialic acid residue by the α2–3 linkage is essential for

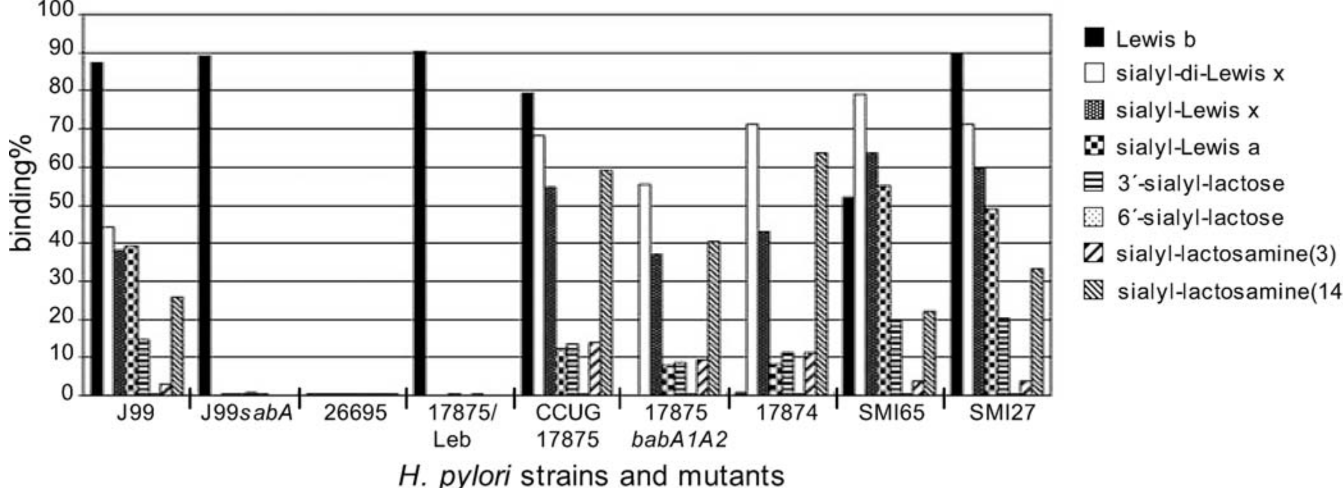

Figure 3. Binding of *H. pylori* Strains and Clinical Isolates to Fucosylated and Sialylated Glycans of Various Complexities

H. pylori reference strains, mutants, and clinical isolates were tested for binding to the fucosylated Leb antigen and to a series of sialylated antigens, all presented by ^{125}I-labeled albumin conjugates. The *y* axis gives the percentage of bound conjugate.
DOI: 10.1371/journal.ppat.0020110.g003

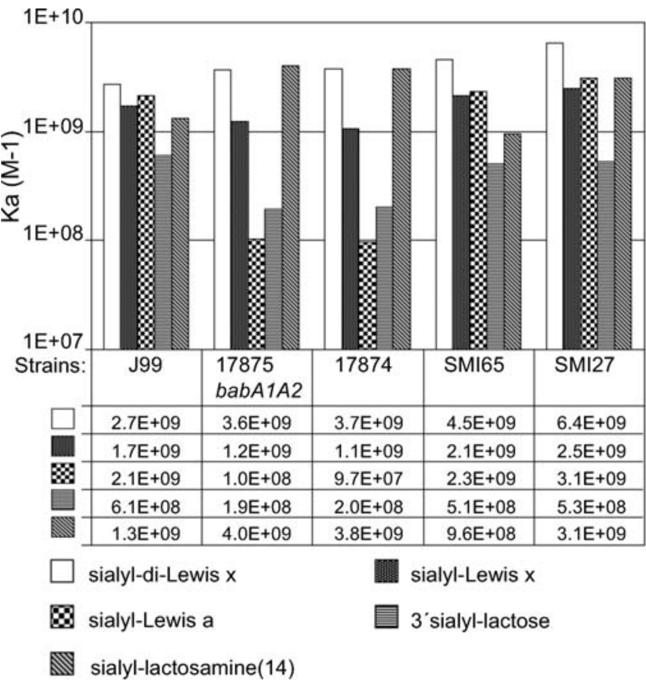

Figure 4. Binding Affinities Analyzed According to Scatchard of *H. pylori* Reference Strains, a *babA* Deletion Mutant, and Clinical Isolates for Sialylated Glycans

DOI: 10.1371/journal.ppat.0020110.g004

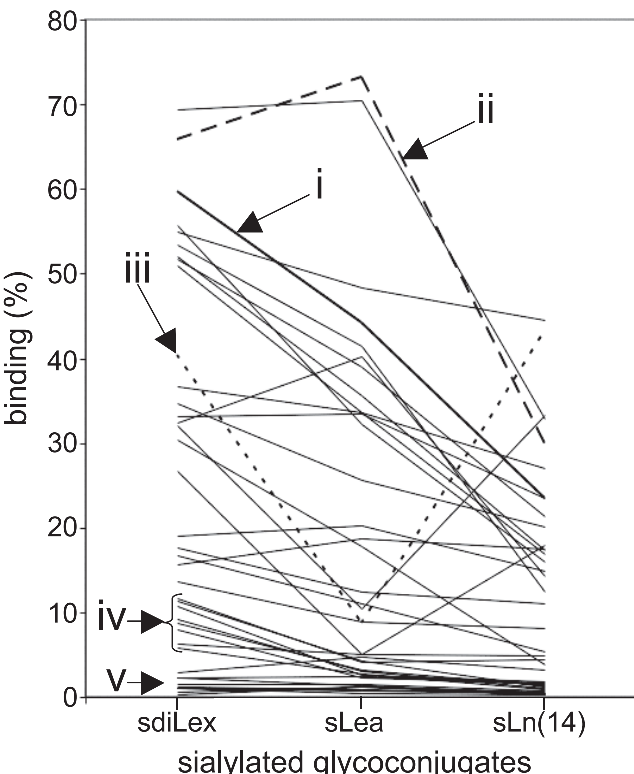

Figure 5. Different Sialyl-Dependent Binding Modes of *H. pylori* Identified by Use of sdiLex, sLea, and sLn Conjugates

A total of 39 Swedish clinical isolates were investigated for detailed sialyl-dependent binding properties. Representatives of the different binding modes for ^{125}I-labeled sialylated glycans are illustrated in the diagram: (i) (in thick line) binds efficiently to all three sialylated glycans with preferential binding to sdiLex; (ii) binds to all three sialylated glycans, with better binding to sLea ("Λ-shaped" hatched line); (iii) binds preferentially to sdiLex and sLn(14) ("V-shaped" dotted line); (iv) binds preferentially to sdiLex but exhibits only modest binding for sLea and sLn(14) sialyl conjugates; and (v) binds modestly for all sialyl conjugates (<5% bound conjugate). The *y* axis gives the percentage of bound conjugate.

DOI: 10.1371/journal.ppat.0020110.g005

SabA-mediated binding both to soluble glycoconjugates and for binding to gangliosides in solid phase. Since binding by SabA to sialyl-lactose was rather weak for all strains, the influence of chain length and steric flexibility in promotion of binding was investigated by use of sialyl-lactosamine (sLn; NeuAcα3Galβ4GlcNAc) attached to albumin, by either the short three-atom [sLn(3)] or the extended 14-atom [sLn(14)] spacer molecules. All strains that bind to sialylated glycans bound ≥4-fold better to sLn(14) than to sLn(3). Strains J99, SMI65, and SMI27 bound strongly to both sLea and sLex, whereas strains 17875, 17874, and the 17875*babA1A2* mutant demonstrated much weaker binding to sLea compared to sLex/sdiLex. In keeping with our previous results, strains 26695, J99*sabA,* and 17875/Leb (a spontaneous mutant which does not bind sialylated antigens) appear to lack binding properties for sLex/sdiLex, sLea [15], and for the sialyl-lactose/lactosamine conjugates tested here.

Analysis of Binding Affinities for Sialylated Antigens

The binding affinities of strains J99, 17874, SMI65, SMI27, and the mutant 17875*babA1A2* for the series of sialylated conjugates described above were analyzed according to Scatchard [37] (Figure 4). Strains J99, SMI65, and SMI27 demonstrated similar profiles in binding affinities for the series of sdiLex, sLex, sLea, and sLn(14) in the range of 9.6×10^8 M^{-1} to 6.4×10^9 M^{-1}. By analogy with the results above, all three strains demonstrated lower binding affinity for sialyl-lactose (5.1×10^8 M^{-1} to 6.1×10^8 M^{-1}). However, sialyl binding is not uniform among strains, since strains 17874 and 17875*babA1A2* demonstrated a different binding pattern, with 10-fold lower affinity for sLea (9.7×10^7 M^{-1} and 1.0×10^8 M^{-1}, respectively) and reduced affinity for sialyl-lactose (2.0×10^8 M^{-1} and 1.9×10^8 M^{-1}, respectively). In the present experimental series, the 17875*babA1A2* mutant exhibited binding affinities, which are approximately 10-fold higher than previously reported [15]. The stronger binding affinity reported relates to the improved bovine serum albumin (BSA) preparation used here as blocking agent, because we found BSA to contain sialyl-competitive constituents (unpublished data). Interestingly, binding to nonsialylated glycans such as Leb was not affected by the improved BSA preparation, which might be due to lack of the human/primate-specific antigen, Leb, in bovine serum constituents. To remove endogenous sialylated glycans, periodate oxidation of the BSA preparation was performed. This treatment resulted in >10-fold higher binding affinities of *H. pylori* for sLex, and consequently, the deglycanated blocking agent was used for all binding analyses in this study.

Polymorphism in Binding to Sialylated Glycans among Clinical Isolates

The 99 Swedish clinical isolates were all analyzed for binding to sdiLex, sLea, and sLn(14) glycans (Figure 5). Of those, 39 (39%) could bind to sialylated glycans, and the great majority, 34 (87%) of 39 of these isolates, could bind all three

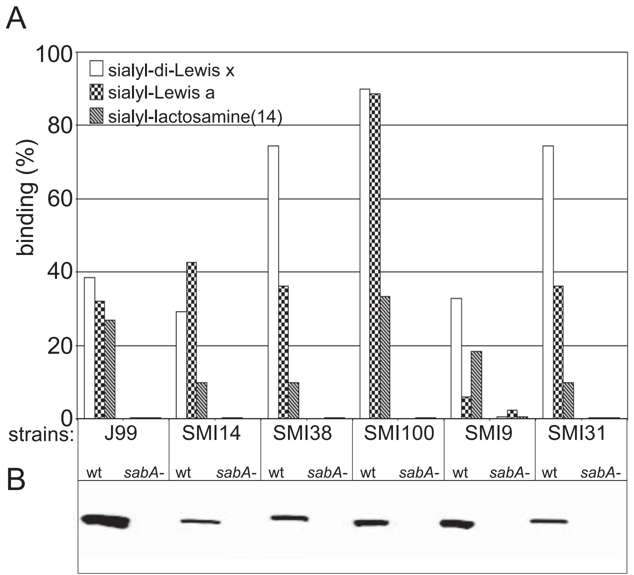

Figure 6. Analyses of SabA-Dependent Binding among Clinical Isolates

(A) Binding characteristics of *H. pylori* clinical isolates and SabA-deficient isogenic mutants were studied by use of soluble ^{125}I-labeled sdiLex, sLea, and sLn(14) conjugates.

(B) Immunoblot analysis of *H. pylori* clinical isolates and mutant strains using SabA antibodies. There were no SabA bands detected among the *sabA* deletion mutants; actually, there were no additional bands on the blots.

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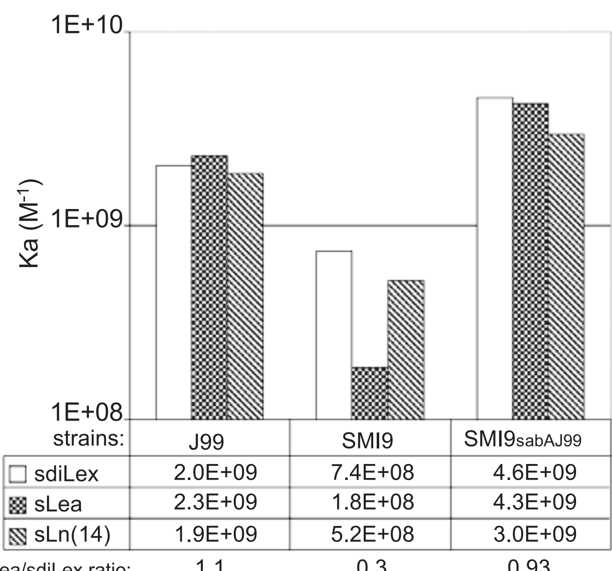

Figure 7. Analyses of Binding Affinities for Sialylated Antigens by SabA from Strain J99 Complementarily Expressed in Background Clinical Strain SMI9

Binding affinities of *H. pylori* strains J99, SMI9, and the complementation mutant SMI9*sabA*$_{J99}$ were analyzed according to Scatchard. Binding affinities and the percentage ratio of sLex/sLea binding affinities are indicated.

DOI: 10.1371/journal.ppat.0020110.g007

glycans. Three isolates exclusively bound sdiLex and two isolates only bound sLea, but isolates with such unusual binding properties were generally poor binders (<1.5% of bound conjugate). Multiple different sialic acid–dependent adherence modes were found among the 39 sialyl-binding isolates, and these were classified as follows: (1) isolates that bind to all three sialylated glycans, with preferential binding to sdiLex (12 of 39 isolates, or approximately 30%); (2) isolates that bind to sLea better than sdiLex and sLn(14) (i.e., strains with an "Λ-shaped" binding mode) (seven of 39 isolates, or approximately 20%); (3) isolates that bind sdiLex and sLn(14) the best, whereas binding to sLea is lower (i.e., isolates with a "V-shaped" binding mode [similar to the binding mode of strain 17874; Figure 3]) (three of 39 isolates, or approximately 10%); (4) isolates that bind sLex but show only modest binding to sLea and sLn(14) (seven of 39 isolates, or approximately 20%); and (5) isolates generally modest in binding (in the interval 1%–5%) for all sialyl conjugates (ten of 39 isolates, or approximately 20%). As shown in Figure 5, preferential binding to sdiLex was the most common binding mode among the clinical isolates tested (24 of 39 isolates, or approximately 60% of strains tested).

Polymorphism in Binding to Sialylated Glycans Is an Inherent Feature of SabA

To test if SabA alone accounts for the polymorphic binding modes to sialylated glycans, *sabA* was deleted in five clinical isolates, which together are representative of the main modes of sialyl-dependent binding (as analyzed in Figure 5). All five isogenic *sabA* deletion mutants were tested both for binding to ^{125}I-labeled sdiLex, sLea, and sLn glycan conjugates and for expression of SabA. The results showed that the series of *sabA* deletion mutants could no longer bind to sialylated antigens, which suggests that SabA is the main factor responsible for polymorphism in binding to sialylated antigens (Figure 6). To thoroughly investigate if the local environment, such as the outer membrane and lipopolysaccharide composition of the individual strain, could influence sialyl-dependent binding properties of SabA, a complementation test was performed. The test was based on the Swedish clinical isolate SMI9 and the reference strain J99, which exhibits low and high proficiency in binding to sLea, respectively, whereas both strains bind sdiLex and sLn the most similarly (Figure 6). The deletion mutant SMI9*sabA*::kan, which cannot bind to sialylated antigens, was used as a background strain for complementation with the *sabA* open reading frame of strain J99. Transformant clones with gained sialyl antigen–binding properties were isolated by an enrichment procedure based on HA and identified by colony screening using SabA antibodies. SabA-positive transformants were analyzed for binding to sdiLex, sLea, and sLn(14) glycans, and the SMI9*sabA*$_{J99}$ complementation mutant was identified. The donor strain J99 exhibits the high sLea/sdiLex binding affinity ratio of 1.1, whereas the recipient strain SMI9 exhibits the low sLea/sdiLex ratio of 0.3. Interestingly, the SMI9*sabA*$_{J99}$ complementation mutant demonstrated a sLea/sdiLex binding affinity ratio of 0.93 (i.e., SabA$_{J99}$ expressed in the background strain SMI9 is most similar in affinity to that of the donor strain J99) (Figure 7). The sdiLex-binding capacity, which reflects the number of functional SabA adhesins on the bacterial surface, remained the same (1.2 nM) in strain SMI9 after introduction of the *sabA*$_{J99}$ gene. In comparison, strain J99 exhibits a binding capacity of 0.5 nM for sdiLex. Sequence analyses demonstrated that full-length J99 *sabA* had recombined into the *sabA* locus of SMI9 *sabA*::kan and formed a

functional *sabA* gene. To verify that the change to a high sLea/sdiLex binding ratio depends exclusively on the $sabA_{J99}$ complementation, a SMI9$sabA_{J99}$::cam deletion mutant was made. Immunoblot and binding analyses of the SMI9$sabA_{J99}$::cam mutant demonstrated both absence of SabA expression and sialic acid binding properties (unpublished data). Taken together, these results conclude that the sialyl polymorphism binding is an inherent characteristic of the SabA adhesin protein itself.

Discussion

Epithelial adherence should benefit *H. pylori* by providing better access to nutrient-rich tissues, and may contribute to delivery of bacterial toxins and various effector molecules through the type IV secretion mechanisms. Tight adherence may also be deleterious for *H. pylori* whenever robust host responses confront the bacterium with bactericidal agents. This suggests that persistence of *H. pylori* in the gastric mucosa depends on the maintenance of a balanced mode of mucosal adherence and the continuous adaptation of its binding properties to functionally match shifts in host glycosylation patterns during chronic inflammation. Mechanisms of rapid adaptation to local inflammation responses may be conferred by rapid on/off phase variation in SabA expression [15] and recombination events involving *babA* and *babB* [38,39].

Once established in the gastric epithelium, *H. pylori* tends to invade the gastric mucosa by slippage through the tight-cell junctions, which is a process that requires reorganization of the cytoskeleton and induced cell morphology [7]. Within the gastric tissue there are several alternative routes available for *H. pylori,* such as SabA-mediated adherence to neutrophils that have invaded the gastric mucosa [5]. Alternatively, *H. pylori* can enter into an intracellular lifestyle, and can invade gastric epithelia cells, whether normal, metaplastic, dysplastic, or cancerous [9], and possibly also gastric stem cells [10]. The inflammatory processes also confers upregulation of sialylated and sulfated carbohydrates in the local vessel lining (i.e., addressins), which act to "home in" on neutrophils from the peripheral circulation (i.e., recruits and activates the inflammatory cells from circulation and directs them to local areas of inflamed and infected tissue [40]). The sialylated parts of the addressins are most similar to the sialylated glycans that are bound by SabA. Thus, invasion of the high-endothelial venule–like vessels might be mediated by SabA-dependent adherence, first to the endothelial lining, and later also to the sialylated erythrocytes. In such a putative scenario, the results presented here are intriguing since systemic dissemination of microbial pathogens might be related to systemic disease. Indeed, chronic dental gum infections, such as periodontal infection in dental pockets, have been shown to frequently leach bacterial cells into circulation, although most bacterial cells are quickly killed by the acquired immunity and complement systems. However, persistent periodontal disease is associated with arteriosclerosis of the carotid arteries and coronary heart disease [41,42]. Interestingly, *H. pylori* infection is also associated with coronary heart disease, suggesting the influence of the disseminated bacterial cells in the long-term development of this pathology [43]. Although the erythrocyte-binding sia-HA phenotype is prevalent among clinical isolates, it has not yet been recognized as a virulence property of *H. pylori,* since sia-HA has not explicitly been shown to correlate with gastric disease [28,44]. However, the prevalence of sialic acid binding may have been underestimated among clinical isolates since: (1) *H. pylori* exhibits on/off phase variation in sialyl binding, and sia-HA could be rapidly lost during passage in culture [15]; (2) almost half of the strains that bound sLex did not cause sia-HA (Figure 2), which suggests that many strains require complex sialylated glycans for binding (such structures are limited on erythrocytes, but are present both in gastric epithelium and on neutrophils [45]); and (3) culture conditions have been shown to influence binding specificity and affinity for sialylated glycoconjugates [34]. In this report, we show that clinical isolates exhibit three distinct HA patterns in response to sialidase treatment (Figure 2): (1) reduced HA titers; (2) increased HA titers; or (3) unaltered HA titers. Reduced HA titers would be the expected effect of sialidase-dependent depletion of sialic acid, whereas an increase in HA titers suggests that depletion of sialic acid may better expose (cryptic) binding sites (i.e., less-accessible glycan epitopes). The third pattern, with no change in HA titers, probably relates to combinations of reduced and increased titers (i.e., strains with yet additional adhesins. The HA properties were found to be highly correlated with sLex binding activity, which implicates SabA as the causative agent in sia-HA. Thus, by the use of deletion mutants, we were able to unambiguously identify SabA as the *H. pylori* sialic acid–dependent hemagglutinin.

To further our understanding of SabA binding, *H. pylori* strains and mutants were tested for binding to sialylated glycans of various lengths and complexities. The results suggest that the NeuAcα2–3Gal disaccharide constitutes the minimal binding epitope for SabA binding, which is in agreement with previous reports on the binding specificity for the *H. pylori* sialyl-dependent hemagglutinin [27]. However, the core chain length was shown to affect SabA-mediated binding to erythrocyte gangliosides, where the NeuAcα2–3-neolactooctaocylceramide bound much better than the shorter NeuAcα2–3-neolactotetraocylceramide. Similarly, semisynthetic glycoconjugates with sialylated glycans presented on 14-atom spacers bound better than short three-atom spacers, which suggests that the sialylated binding epitope is best presented by extended and flexible core chains. Furthermore, all strains demonstrated increased binding for sLex, sdiLex, and sLea compared to sialylated structures lacking fucose constituents. These results are in keeping with our previous results on high-affinity binding of SabA to extended gangliosides with repetitive Lex motifs [15]. In the present study, we also showed by protease treatment analysis of host cell surfaces that *H. pylori* HA is mainly mediated by binding to glycosphingolipids. Nevertheless, the sialylated protein (albumin) conjugates used in this study are also efficient receptors for SabA-mediated *H. pylori* binding. This might in part relate to the uniform coating and presentation of sialylated glycans on the globular albumin molecule, which mimic the host cell–surface presentation of receptor glycans. In addition, the soluble albumin conjugates are probably more efficient for bacterial binding due to pretty flexible presentation of the sialylated antigens compared to bacterial binding to surfaces presented glycolipids (i.e., solid-phase interactions). Thus, strong and multivalent ("Velcro"-type) binding to sialylated epitopes on glycosphin-

golipids (gangliosides) with extended core chains would promote membrane-tight binding of SabA during experimental HA and in the inflamed gastric epithelium.

In the present study, the great majority of sialyl-binding *H. pylori* isolates bound the full series of sdiLex, sLea, and sLn glycans. This is a distinct difference compared to previous results, where merely half of sLex-binding strains also bound sLea [15]. The higher prevalence of sLea binders reported here is probably best explained by the presence of competitive sialylated antigens in the albumin-based blocking agent used previously. This is also supported by the 10-fold increased binding affinity for sLex, 1×10^9 M^{-1}, revealed by strain 17875*babA1A2* (Figure 4). Interestingly, clinical isolates demonstrated several distinct binding modes for sialylated glycans, although most isolates bound best to sLex. The differences in binding affinities to various sialylated glycans relate both to complexities in fucosylation and to the type of lacto-series core chains. Subtle differences in binding specificities have been described for urinary tract infectious Galα4Gal-binding P-fimbriated *E. coli.* For urinary tract infectious isolates the length of the globoseries glycolipids, required for bacterial binding, sorted the cognate adhesins into different functional subtypes that recognize human or canine kidney tissue [46,47]. In addition, single amino acid changes in type-I fimbriated *E. coli* urinary tract infectious isolates can change the normal binding mode for antennary mannosylated structures into a high-affinity binding mode that also accepts mono-mannosylated structures [48].

Hence, detailed differences in binding properties often relate to the adhesin polypeptide itself, but the binding mode can sometimes be distinctly influenced by physical constraints imposed by associated proteins in the local environment (e.g., the detailed binding specificity of *Salmonella* type I fimbriae is dependent on the fimbrial shaft on which the FimH adhesin is presented [49]). In contrast to pilus-associated adhesins, SabA exhibits a C-terminal putative ß-barrel domain, and hence is most likely instead a membrane-integrated protein. Our studies point to the fact that the detailed binding properties of SabA described here, similar to BabA [14], are inherent features of the adhesin proteins themselves.

The polymorphic binding to sialylated conjugates probably relates to small differences in SabA, which is similar to BabA in that positive selection for nonsynonymous codon substitutions have generated variant BabA adhesins that demonstrate specialist and generalist binding modes for ABO blood group antigens [14]. The high mutation rates in *H. pylori* [50,51] will promote formation of derivative strains with modified binding properties. Polymorphic binding properties could be of utmost importance during mixed infections, when recombination and exchange of genetic information might promote formation of transformants with chimeric binding properties that together behaves like a quasi-species. The occurrence of *H. pylori* strains that express SabA adhesins with different subtypes of binding modes as described here could therefore reflect such ongoing molecular evolution.

H. pylori infection and gastritis have been found to enhance gastric mucosal expression of the inflammation associated sLex/sLea antigens [15]. These sialylated glycans are dynamically expressed in competition with fucosylated blood group antigens [52]. Furthermore, malignant transformation has been reported to confer a pronounced expression of Lea, sLea, and sdiLex in the gastric mucosa [19,53]. Such changes to the differentiation programs of the gastric epithelial cell lineages will promote expression of a wide range of sialylated antigens. Taken together, these reports suggest that *H. pylori* infection and the associated chronic inflammation responses continuously change the availability of sialylated glycans of different complexities. Such changes in the gastric sialylation patterns would select for SabA clones that evolve with new or modified binding properties for sialylated epitopes. Thus, the bacteria–host crosstalk selects for polymorphic clones with optimal fitness for both the sialyl archipelago of the local environment (gastric mucosa) and the individual's phenotype for balanced, lifelong infection.

Materials and Methods

Bacterial strains. The *H. pylori* strains used in this study were CCUG17875, CCUG17874 [54], 26695 [55], J99 [2], the *sabA* deletion mutant J99*sabA*(JHP662)::cam, the *babA* deletion mutant J99*babA*::cam, and the *babA* "double" mutant 17875*babA1::kan babA2::cam* (the series of mutants are abbreviated J99*sabA,* J99*babA,* and 17875*babA1A2,* respectively). The 17875/Leb strain is a spontaneous mutant that binds Leb but does not bind to sialylated antigens [15]. The panel of 99 clinical *H. pylori* isolates (including the series of SMI strains) came from Uppsala University Hospital, Sweden, and have been described previously [54]. Bacteria were grown on Brucella agar supplemented with 10% bovine blood and 1% IsoVitalex (Svenska LABFAB, http://www.labfab.se), for 43–48 h at 37 °C in 10% CO_2 and 5% O_2 before harvest in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 1% BSA (Sigma-Aldrich, http://www.sigmaaldrich.com). *E. coli* strains HB101/pPAP5 [47] and HB101/pAZZ50 [56] were cultured overnight on Luria broth plates supplemented with chloramphenicol and tetracycline.

Biopsies. Gastric biopsies harvested at endoscopy in human and monkeys were fixed in Z-fix (10% paraformaldehyde + 1% ionized Zn; Anatech LTD, http://www.anatechltd.com), dehydrated, and embedded in paraffin. The protocol involving human subjects was approved by the Institutional Review Boards of the Veterans Affairs New York Harbor Health Care System and the Uniformed Services University of the Health Sciences, and written informed consent was obtained from all patients before study entry. All animal experiments were approved by the Armed Forces Radiobiology Research Institute Institutional Animal Care and Use Committee and monitored and reapproved at yearly intervals. All experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, (Washington, D. C.: National Academy Press, 1996).

In situ hybridization. Sections (5 μm) were processed for in situ hybridization [9]. Sections were first deparaffinized in xylene, rehydrated in graded ethanol, and treated with proteinase K. They were then covered with hybridization mixture as described [9]. A specific cDNA probe was designed using a sequence database for *H. pylori 16S rRNA* [9], and the 5′ end of the oligonucleotide was labeled with biotin. The probe was denatured by heating for 10 min at 65 °C; a drop of probe was then placed on the tissue, and the reaction was incubated overnight at 37 °C for 18 h. The unbound probe was removed by successive washes in decreasing concentrations of Na citrate at room temperature and at 65 °C for the last wash. The biotin-labeled probe was detected by streptavidin-conjugated alkaline phosphatase (2 h of incubation at room temperature), and a chromogenic substrate (nitro-blue-tetrazolium, NBT/BCIP kit; Vector Labs, http://www.vectorlabs.com). After washing, sections were counterstained with Nuclear Fast Red and mounted with permount. Positive controls were as described [9] and included *H. pylori* pure cultures streaked onto precleaned microscope slides. Negative controls included *E. coli* and *Shigella flexneri* cultures, and gastric biopsy specimens from a 62-y-old *H. pylor*i–negative patient with dyspepsia were also used as negative controls [9]. Finally, control for nonspecific binding was performed by using sense instead of antisense probe, hybridization buffer instead of antisense probe, unlabeled antisense probe, digoxigenin- or biotin-labeled probe for the scorpion *Buthus martensi* Karsch neurotoxin sequence (5′-GGC CAC GCG TCG ACT AGT AC-3′), RNase A pretreatment (Roche,

http://www.roche.com), DNase I pretreatment (Roche), and RNase plus DNase I pretreatment [9].

Epoxy (Spurr low viscosity) semithin sections stained with toluidine blue. Biopsies were processed for transmission electron microscopy using the conventional standard method [9]. Semithin sections of 0.5 μm were stained with 1% toluidine blue.

Image acquisition. Sections were viewed using a light microscope Nikon Eclipse E300 (http://www.nikon.com) and pictures were captured and digitized using a QCapture camera (QImaging, http://www.qimaging.com) and Micropublisher 5.0 RTV (QImaging).

HA conditions and reagents. Fresh human blood from a healthy donor of blood group A phenotype was used for the HA assays. The erythrocytes were washed twice with PBS. A 20% erythrocyte suspension was treated at 28 °C with 0.05 or 0.1 mg/ml trypsin (Sigma, http://www.sigmaaldrich.com) for 2 h at neutral pH [57] followed by 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma) for 15 min, and then gently washed five times with PBS. A 4% suspension of trypsin-treated or untreated erythrocytes was incubated with sialidase type VI from *C. perfringens* (Sigma) at 0.1 U/ml [26]. After washing, a 0.75% erythrocyte suspension was mixed with a 2-fold dilution series of *H. pylori* for estimation of sia-HA titers (i.e., the reduction in HA titers as a result of sialidase treatment of erythrocytes). Round-bottomed enzyme-linked immunosorbent assay (ELISA) plates were used for the analysis, and the aggregation of erythrocytes was determined visually after 1 h at room temperature (23 °C).

Neoglycoproteins. All glycan conjugates used were semisynthetic glycoproteins constructed of purified or chemically synthesized oligosaccharides conjugated to either human serum albumin (HSA) or to BSA (see Table 1). Leb, sLex, sdiLex, sLea, 3′-sialyl-lactose, 6′-sialyl-lactose (IsoSep AB, http://www.isosep.com), and sLn (with three-atom or 14-atom spacer, sLn(3) and sLn(14), respectively) glycoconjugates (Dextra Laboratories, http://www.dextra-labs.co.uk) were labeled with ^{125}I by the chloramine-T method [54]. The glycan densities of the conjugates were 24 mol Leb oligosaccharides/mol HSA, 13 mol sLex oligosaccharides/mol HSA, 11 mol sdiLex oligosaccharides/mol HSA, 12 mol sLea oligosaccharides/mol HSA, 6 mol 3′-sialyl-lactose oligosaccharides/mol HSA, 15 mol 6′-sialyl-lactose oligosaccharides/mol HSA, 11 mol sLn(3) oligosaccharides/mol BSA, and 11 mol sLn(14) oligosaccharides/mol BSA.

Periodate oxidation of BSA blocking agent to destroy competitive carbohydrate receptors for *H. pylori* binding. BSA (A3294; Sigma), 15% wt/vol in Milli-Q water (MQ; Millipore, http://www.millipore.com), was treated with 10 mM periodate for 1 h at pH 4.5. The reaction mixture was then reduced with 20 mM $Na_2S_2O_5$ (pH 6.7). Reagents were removed by dialysis against MQ followed by PBS containing 0.05% Tween 20 (PBS-Tween). Treated BSA was stored in aliquots at −20 °C. BSA treated by periodate oxidation did not quench sialyl binding, whereas the untreated BSA preparation substantially reduced binding of the 17875*babA1A2* mutant.

Analysis of *H. pylori* binding to soluble neoglycoproteins by radioimmunoassay. For radioimmunoassay analyses, 1 ml of bacteria (OD 600 nm = 0.1) was mixed with 5 ng of ^{125}I-labeled conjugate. After 2 h at room temperature, the bacteria were pelleted and bound conjugate was measured by gamma scintillation counting (Wallac, http://las.perkinelmer.com) [54]. Binding experiments were carried out in duplicate. To test the periodate-treated BSA for presence of competitive carbohydrate receptors, bacteria of strain SMI65 (OD 600 nm = 0.1) were mixed with 2 ng of ^{125}I-labeled sdiLex conjugate and a dilution series of 0, 0.2, 0.4, 0.6, 0.8, and 1% (wt/vol) periodate-treated or untreated BSA in PBS. After 2 h of incubation at room temperature, bacteria were pelleted and bound conjugate was quantified as described above. The results revealed that periodate-treated BSA did not inhibit sLex binding, whereas the untreated BSA showed strong inhibition. The full series of binding experiments reported in this study have been performed by use of this deglycanated BSA-blocking agent.

Affinity adsorption of SabA to erythrocyte surfaces. Bacteria were harvested from overnight cultures, washed once with PBS, and then treated with 500 μl deionized water for 15 min at room temperature with gentle shaking. After centrifugation for 30 min at 10,000*g* at 4 °C, 10× PBS was added to the supernatant to bring it to 1× PBS. A total of 200 μl of 4% erythrocyte suspension (with or without prior sialidase treatment) was mixed 1:1 with bacterial protein extract for 1 h at room temperature. The erythrocytes were washed three times with PBS, mixed with SDS-PAGE sample buffer, and heated for 5 min at 95 °C. Electrophoresis was carried out using a 7.5% Tris-HCl Ready gel (Bio-Rad Laboratories, http://www.bio-rad.com). For immunoblot analysis, proteins were transferred to immunoblot polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked for 1 h in PBS-Tween containing 5% nonfat dried milk. Antibodies were raised in rabbit against full-length recombinant SabA as previously described for BabA [58]. Blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit antibodies (Dako, http://www.dako.com) diluted in PBS-Tween containing 1% nonfat dried milk, and finally developed with SuperSignal West Pico Chemiluminiscent Substrate (Pierce, http://www.piercenet.com).

Glycosphingolipids. Total non-acid and acid glycosphingolipid fractions from human erythrocytes were obtained by standard procedures [59]. Individual glycosphingolipids were isolated by acetylation of total glycosphingolipid fractions and repeated chromatography on silicic acid columns. Acid glycosphingolipid fractions were separated by DEAE-Sepharose chromatography, followed by repeated silicic acid chromatography, and final separation was achieved by high-performance liquid chromatography. The identity of the purified glycosphingolipids was confirmed by mass spectrometry [60], proton nuclear magnetic resonance spectroscopy [61], and degradation studies [62,63]. A detailed description of the isolation and characterization of the *H. pylori*–binding gangliosides has been given elsewhere [45].

Chromatogram binding assay. The conditions used for culture, ^{35}S-labeling of *H. pylori,* and the chromatogram binding assays were as described previously [36]. Mixtures of glycosphingolipids (40 μg/lane) or pure compounds (1–4 μg/lane) were separated on aluminum-backed silica gel 60 high-performance thin-layer chromatography plates (Merck, http://merck.com) using chloroform/methanol/0.25% KCl in water (50:40:10 by volume) as a solvent system. Thereafter, a suspension of ^{35}S-labeled bacteria (diluted in PBS to 1×10^8 CFU/ml and $1–5 \times 10^6$ cpm/ml) was sprinkled over the chromatograms and incubated for 2 h at room temperature. After washing with PBS and drying, the thin-layer plates were autoradiographed using XAR-5 X-ray film (Eastman Kodak, http://www.kodak.com).

Analysis of binding affinities for soluble glycoconjugates. The affinity for *H. pylori* SabA-mediated binding to sialylated glycoconjugates was measured by Scatchard analysis [37]. Each *H. pylori* strain exhibits distinct binding affinities for sialylated receptor structures. Thus, for the affinity analyses, the bacterial cells were diluted to ascertain a free-to-bound ratio of conjugate binding close to 1.0 (equivalent to an equilibrium of ∼50% bound glycoconjugate). Unlabeled glycoconjugate was then added in a dilution series of seven different concentrations, where the highest concentration was predicted to block approximately 80% of glycoconjugate binding. The bacterial cell–glycoconjugate mixture was incubated at room temperature for 2 h in PBS-Tween with 1% of the periodate-treated BSA as blocking agent. Bound conjugate was analyzed as described above by gamma scintillation counting for 5 min.

Construction of *sabA* deletion mutants. To construct SMI14*sabA*::kan, SMI38*sabA*::cam, SMI100*sabA*::kan, and SMI31*sabA*::cam *sabA* deletion mutants, the Swedish clinical isolates SMI14, SMI38, SMI100, and SMI31 were first single-colony purified and transformed with *sabA*::kan or *sabA*::cam deletion vectors (previously described in [15]). For construction of SMI9*sabA*::kan, *sabA* was first PCR-amplified using the primers M3F and M4R and cloned in the pBluescript $SK^{+/-}$ EcoRV site. The plasmid clone was then linearized with M7F and M5R and ligated with the *kanR* gene, as described in [54]. *H. pylori* transformants were analyzed for binding to ^{125}I-labeled sLex conjugate and for expression of SabA using anti-SabA antibodies, as described above. The primer sequences were as follows: M3F (5′-CGCTAGTGTCCAGGGTAAC-3′); M4R (5′-TTGATCGTAAGG-CAGTGTGATA-3′); M7F (5′-TCCCTAAAGATCAGTATCGT-3′); and M5R (5′-CCGCGTATTGCGTTGGGTAG-3′)

Construction of SMI9 *sabA*$_{J99}$ recombinants. The *sabA* gene of strain J99 was amplified by PCR using primers J99-4F and J99-7R. The resulting DNA fragment was transformed into strain SMI9*sabA*::kan. Transformants expressing recombinant SabA were isolated by the HA method as described below. Sequencing of *sabA* was performed using primers *sabA*-278F and *sabA*-1136R. The primer sequences were the following: J99-4F (5′-GAATACGCAATCTTGTGGAGT-3′); J99-7R (5′-CCAAATCACCCAATTACTTTG-3′); *sabA*-278F (5′-TACAACAG-CACCACCCAA-3′); and *sabA*-1136R (5′-CATCTTTAGCCACGCT-TAA-3′).

HA method for enriching sialic acid–binding *sabA* recombinants. For isolation of sialyl-binding transformants fresh human blood from a healthy donor was used. The erythrocytes were washed three times with PBS, and a 4% erythrocyte suspension was added onto 1 ml of bacterial transformation mixture ($OD_{600} = 1$). The bacterial-erythrocyte suspension was incubated with gentle rocking for 30 min at 37 °C in 10% CO_2 and 5% O_2. For separation of transformants with gained erythrocyte-associated (sialyl-binding) binding properties from the nonbinding (parent) SMI9*sabA* deletion mutant,

aggregated erythrocytes were allowed to sediment for 25 min. After removal of the supernatant and three rinses with PBS, 200 µl Brucella broth was added and the suspension was spread on a Brucella agar plate. Clones that express full-length SabA were identified by colony screening as described below. The HA and colony-screening procedure was repeated three times for efficient enrichment of clones of background strain SMI9, which had gained in complementary expression of SabA from donor strain J99.

Colony screening for sialic acid–binding *sabA* recombinants. For identification of SabA-expressing clones the bacteria were spread in serial dilutions onto Brucella agar plates and cultured until single colonies appeared. The bacterial colonies were printed onto nitrocellulose membranes (Bio-Rad), which subsequently were soaked in boiling hot 1× sample buffer (4 ml 10% SDS, 1.6 ml Tris [pH 6.8], and 14.4 ml MQ H_2O, with 500 µl β-mercaptoethanol) for 5 min. The membranes were blocked overnight in TBS with 0.05% Tween 20 (TBS-Tween) containing 5% nonfat dried milk at 4°C. SabA-expressing clones were detected by using anti-SabA antibodies (described above) and horseradish peroxidase–conjugated goat anti-rabbit antibodies and finally visualized by using 4-chloro-1-naphtol (4C1N) tablets (Sigma).

Construction of SMI9*sabA*$_{J99}$ *sabA* deletion mutants. In order to construct SMI9*sabA*$_{J99}$::cam, SMI9*sabA*$_{J99}$ was transformed with a *sabA*::cam deletion vector as described [14]. Chloramphenicol-resistant transformants were analyzed for binding and expression of SabA as described above.

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Competing interests. In 2002 TB filed a patent application for the use of SabA as a vaccine candidate, International PCT pending number PCT/SE02/00301/ *Helicobacter pylori* sialic acid binding adhesin, SabA, and *sabA* gene.

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