Role of *Helicobacter pylori* SabA Adhesin During Persistent Infection and Chronic Inflammation

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Supplementary material

MATERIALS AND METHODS

Supplemental Methods 1. For Adherence In Situ (AIS) (*S1*), biopsies were obtained from Drs. Ray Clouse, Washington University Medical Center, St. Louis, David Graham, VA Medical Center, Houston, and Intissar Anan, Umeå University. Gastric tissue sections from Leb mice and FVB/N mice were from Drs. Jeff Gordon and Per Falk, Washington University. To inhibit AIS (*S1*) *H. pylori* was labeled with FITC and mixed with Leb conjugate (10 µg/mL) or sialyl-Le x/a-conjugates (20 µg/mL) (IsoSep AB, Tullinge, Sweden). Alternatively, histo sections were pre-treated with the Leb mAb or the FH6 mAb (which recognizes the sdiLex antigen), at 1:100 dilution for 3 h. Reduction in bacterial binding was estimated by the number of adherent bacteria under 200X magnification. Each value is the mean +- SEM of 10 different fields, with significance evaluated with the Student's t-Test.

Supplemental Methods 2. *H. pylori* strains 26695, CCUG17875 (designated 17875), the construction of the 17875*babA2*::cam-mutant, and the G27*cag*PAI-deletion mutant, were described in (*S2*). The construction of 17875*babA1*::kan *babA2*::cam (abbreviated the *babA1A2*-mutant), *sabA*(JHP662)::cam and the *sabB*(JHP659)::cam mutants were as described in (*S2*) with minor modifications (see Supplemental Methods 3). J99 was kindly provided by Drs. Tim Cover, John Atherton and Martin Blaser. The 77 cag^+ and 12 cag^- Swedish isolates, including SMI65, were kindly provided by Dr. Lars Engstrand, and 6 Italian $cagA^-$ strains were described in (*S2*). Strain WU12 was from a patient with gastritis (Fig 1A-M), treated at Washington University Medical Center, St.

Louis. Strain J166 was described in (*S3*). The 17875/Leb strain, a spontaneous sLex$^-$ variant was isolated by screening single colonies of strain 17875. Bacteria were grown for 2 days on Brucella agar medium at 37°C, under 10 % CO_2 and 5% O_2, for optimal binding.

Supplemental Methods 3. Construction of *babA1A2*-, *sabA*-, *sabB*-, and *babA/sabA*-deletion mutants. The 17875*babA1*::kan *babA2*::cam, J99*sabA*(JHP662)::cam, J99*sabB*(JHP659)::cam, and the J99*babA*::cam *sabA*::kan-mutant strains were constructed as described in (*S2*), with the following modifications;

(3A) Strain CCUG17875 was used for construction of 17875*babA1*::kan *babA2*::cam (abbreviated the *babA1A2*-mutant). The *babA1* gene was amplified using the F44 (forward) and R44 (reverse) primers, cloned into the pBluescript SK+/- EcoRV site (Stratagene, La Jolla CA), linearized with primers R41+F38, ligated with the KanR cassette from pILL600 (*S4*), and used to transform the original 17875*babA2*::cam mutant (*S2*). The transformants were analyzed by PCR using upstream primers F2 (*babA2*) or F44 (*babA1*) in combination with the R11 *babA* primer (located in the segment replaced by the KanR cassette). The 17875*babA1*::kan *babA2*::cam (double)-mutant (abbreviated the *babA1A2*-mutant) could not be amplified with either primer pair.

F2: CTTAAATATCTCCCTATCCC
R41: GCGAGCCTAAAGTTAATGA
F38: ACGTGGCGAACTTCCAATTC
F44: CAGTCAAGCCCAAAGCTATGC
R11: CGATTTGATAGCCTACGCTTGTG
R44: CTTAAAGGGATAGGAAGCGCT

(3B) Strain J99 was used for construction of the *sabA*(JHP662)::cam and the *sabB*(JHP659)::cam mutants. *SabA* was PCR-amplified using the primers F18 and R17 and cloned in pBluescript SK+/- EcoRV site. The plasmid clone was linearized with R20 and F21 and ligated with the *camR* gene, described in (*S2*). *SabB* was amplified using primers F16 and R15, and cloned in pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), linearized with HincII and ligated with the *camR* gene. *H. pylori* transformants were analyzed for binding to ^{125}I-labeled sLex conjugate. The location of the *camR* gene in *sabA* and *sabB* was analyzed using primers R17+F18 and F16+R15, respectively, which verified

that loss of binding was dependent on the *cam*-insertion and independent of spontaneous OFF-(phase)-variation in sLex binding.

R15: CTATTCATGTTTACAATA
F16: GGGTTTGTTGTCGCACCACTAG
R17: GGTTCATTGTAAATATAT
F18: CGATTCTATTAGATCACCC
R20: AGCGTTCAATAACCCTTACAGCG
F21: GATTTAAATACTGGCTTAATTGCTCG
BS22: CGCTTAAAGCATTGTTGACAGCC

(3C) Strain J99 was used for construction of the J99*babA*::cam *sabA*::kan-(double)-mutant. For the construction of the *babAsabA*-mutant strain, *sabA* was first cloned in the pBluescript vector and linearized as described above, and then ligated with the KanR cassette (see above). For the construction of the J99*babA* and *babAsabA*-double mutants the J99 and J99*sabA*::kan strains were transformed with the *babA* deletion vector described in (*S2*). The transformants were analyzed by PCR using primers F33 + R34. The correct J99*babA*::cam and the J99*babA*::cam *sabA*::kan-(double)-mutant could not be amplified with the primer pair.
F33: ATCCTTTCATTAACTTTAGGATCGC
R34: TTGAGCGCTATCAGGCACAC

Supplemental Methods 4. Retagging (of SabA) and Identification of *sabA*, the Gene which Corresponds to the Sialic acid binding Adhesin, SabA.

For identification and purification of the SabA-adhesin, the Retagging technique was used, i.e., similar to the identification of the Leb antigen binding BabA-adhesin (*S2*), with some modifications. Here, the *babA1A2*-mutant was incubated with Sulfo-SBED (Pierce, Rockville, IL.) attached to sLex-conjugate. The crosslinker was activated by overnight UV irradiation, and biotin-(Re)tagged proteins were purified with magnetic streptavidin beads. The 66kDa band purified was digested with Trypsin (seq grade, Promega, USA) and four purified peptides were identified by mass spectrometry based on peptide masses and sequences. MALDI-TOF-MS on a Tof-Spec E mass spectrometer (Micromass, Manchester, England) (*S 5*), and ProFound (www.proteometrics.com) was used for matching of the four peptide masses using NCBI database. Peptides 1 and 2 (see below) match the JHP662 (*S6*)/HP0725 (*S7*) gene. Peptides 3 and 4 also matched the JHP659 (*S6*)/HP0722) (*S7*) gene. Peptide identities were validated by ESI-MS/MS sequencing on a Q-Tof instrument (Micromass), using the nanospray source (*S8*).

Mascot (www.matrixscience.com) identified the peptide sequences (numbered according to the JHP662 peptide sequence:
(1) aa68-QSIQNANNIELVNSSLNYLK-aa87
(2) aa301-DIYAFAQNQK-aa310
(3) aa500-YYGFFDYNHGYIK-aa512
(4) aa620-IPTINTNYYSFLGTK-aa634

Supplemental Methods 5. Presence of the *sab*A gene.
The presence of *sabA* in clinical *H. pylori* isolates was analyzed by PCR using primer pairs F1 + 5R, and 3F + 1R.
1F: CTCTAGCAATGTGTGGCAG
3F: CGCTAGTGTCCAGGGTAAC
1R: GCGCTGTAAGGGTTATTGAAC
5R: CCGCGTATTGCGTTGGGTAG

Supplemental Methods 6. *H. pylori* overlay to HPTLC separated glycosphingolipids (GSLs).
Mixtures of GSLs (20-40 μg/lane) or pure compounds (0.002-4 μg/lane) were separated on silica gel 60 HPTLC plates (Merck) and chemically detected with anisaldehyde. Binding of ^{35}S-labeled *H. pylori* to TLC separated GSLs was according to (*S9*). The KM-93 mAb (Seikagaku Corp., Tokyo, Japan) was detected using ^{125}I -rabbit anti mouse antibodies from Dako, Glostrup, Denmark.

Supplemental Methods 7. Isolation and identification of the sdiLex GSL
The *babA1A2*-mutant strain was used to purify a high affinity GSL with receptor activity from human gall bladder adenocarcinoma tissue, which is a rich source of extended □1,3-fucosylated gangliosides (*S10),* by chromatography on silicic acid columns, as described (*S11*). Fractions were tested for *babA1A2*-mutant binding. Desialylation was performed by mild acid hydrolysis in 1% (v/v) acetic acid for 1 hr at 100°C. Negative ion FAB mass spectra were recorded on a JEOL SX-102A mass spectrometer (JEOL, Tokyo, Japan). The ions were produced by 6 keV xenon atom bombardment, using triethanolamine as matrix and an accelerating voltage of -10 kV. 1H NMR spectra were obtained on a Varian 600 MHz machine at 30°C using dimethylsulfoxide-d6/D2O (98/2) as solvent. The NMR spectrum was compared with earlier published data for

this structure recorded at 55°C (*S12*) and corresponding structural elements of the Y-6 glycosphingolipid recorded at 30°C (*S13*). The *H. pylori* binding GSL was then identified by negative ion FAB mass spectrometry and ^{1}H NMR as NeuAc□2.3Galß1.4(Fuc□1.3)GlcNAcß1.3Galß1.4(Fuc□1.3)GlcNAcß1.3Galß1.4Glcß1Cer (Figs. 2B: lane 6, and 2C) (i.e., the sialyl-dimeric-Lewis x glycosphingolipid), abbreviated sdiLex.

Supplemental Methods 8. RIA and Scatchard analyzes

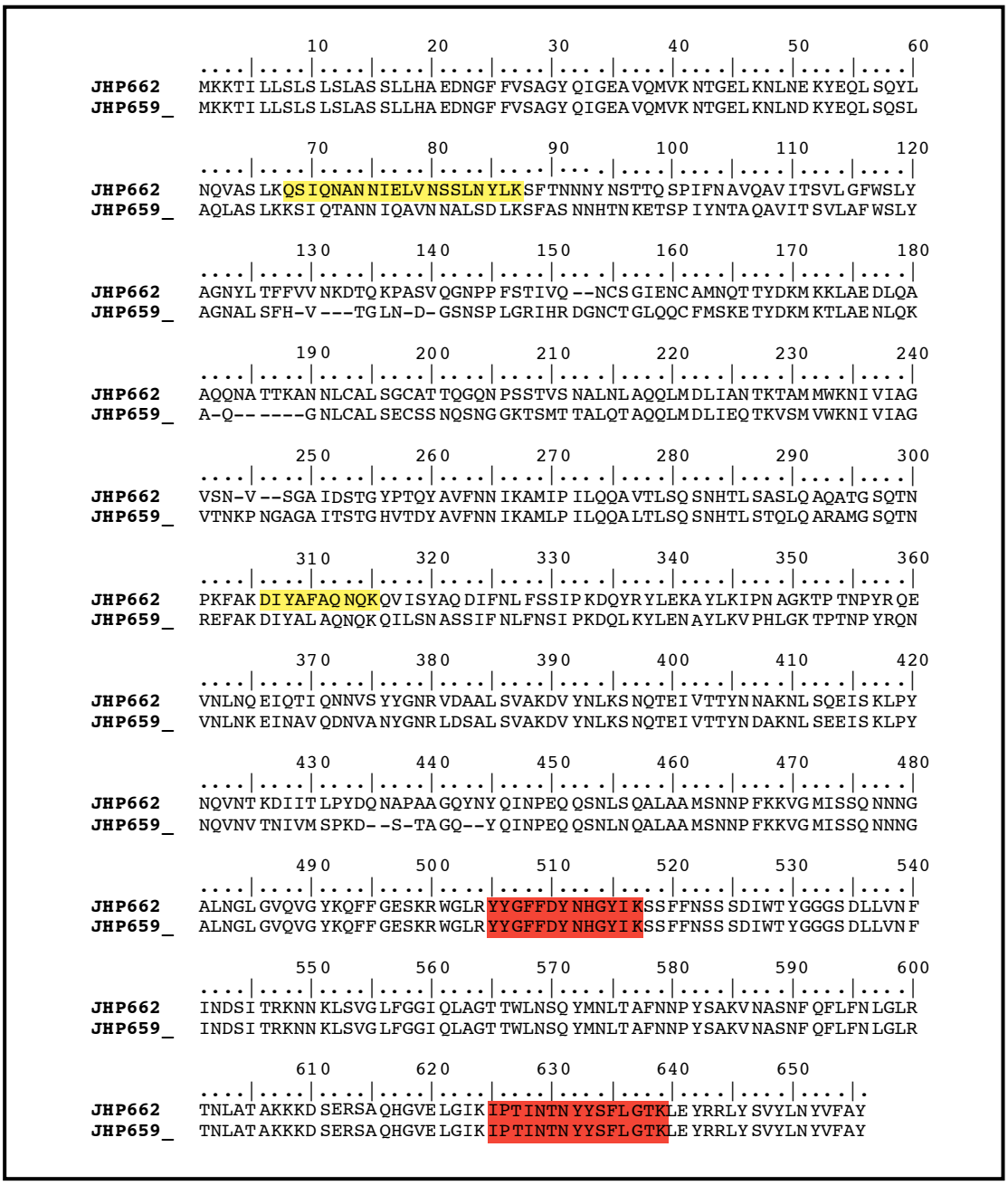
SLea, sLex, and Leb glycoconjugates (IsoSep AB), and sdiLex-glycoconjugate synthesized according to (*S14*), (all based on albumin), were labeled with ^{125}I by the chloramine T method and mixed with *H. pylori* bacteria for binding and Scatchard analyzes (*S15*), essentially as described in (*S2*). The binding experiments were performed in triplicates.

Supplemental Methods 9. Gastric biopsies analyzed for *H. pylori* binding activity and inflammatory scores

Gastric biopsies from 29 individuals without dysplasia were H/E-stained and evaluated for lymphocyte/ plasmacell infiltration. PMN cell infiltration was evaluated from rare PMNs only found in the lamina propria, up to pit abscesses. SLex antigen expression was analyzed by the KM-93 mAb in the surface mucous cells/gastric pit region. Histological gastritis was also graded. *H. pylori* was Genta-stained and quantified. AIS was scored by the number of bacteria/gastric pit region. Each value is the mean of 5 different fields. All scores are available in Table S2A. Adherence scores were correlated with infiltration scores of lymphocytes/plasmacells, PMNs, staining scores by sLex mAb, gastritis and also *H. pylori* infections, and statistically analyzed according to Pearson.

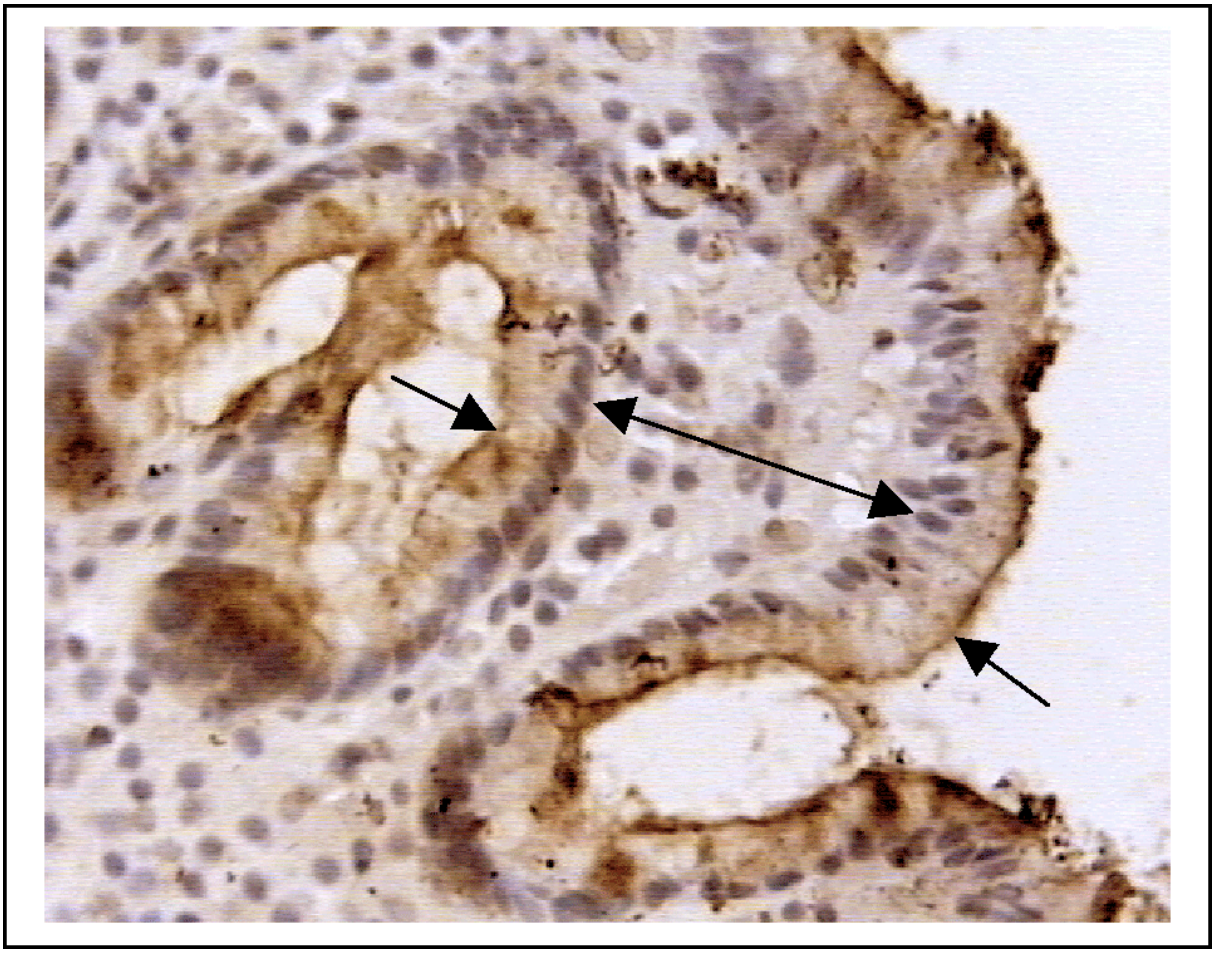
Supplemental Methods 10. Gastric biopsies of monkey E6C were taken 6 months post-cure (for Figs. 4A and 4B). Final biopsy samples from E6C were taken 9 months after established re-infection (Figs. 4C-4F) (*S3*). Biopsies were stained with the KM-93 mAb, and AIS by the *babA1A2*-mutant was analyzed as described in Supplemental Methods 1.

Supplemental Figure 1. Alignment of JHP662/ JHP659,



All four peptides were aligned with the JHP662 gene (4 peptide match), while only (2 (red) peptides matched) the JHP659 gene. Thus, the results suggest that *sabA* correspond to the JHP662 gene (*S6*)/ HP0725 (*S7*) gene.

Supplemental Figure 2. Apical localization of epithelial sLex antigens in inflamed gastric epithelium



Sialylated glycoconjugates, such as sLex are rare in healthy human stomachs, but sialylation is upregulated during chronic inflammation and gastritis. The cellular (topical) localization of the sialylated antigens was investigated using a sLex specifik mAb. In the figure (400x magn.), the sLex antigen is visualized in dark staining lining the apical surfaces (single arrows). In contrast, the clue of nucleus (which is located towards the basolateral side of the cells) stained with Mayer´s hematoxylin (double arrow). The results suggest that the sLex-antigen is expressed at the apical surfaces of the epithelial cells, in response to *H. pylori* adherence and stimuli.

For immunohistochemistry, sections (5 □m) of inflamed gastric mucosa were stained with the sLex specific mAb ((KM-93), Seikagaku Corp., Tokyo, Japan), in 1:10 dilution, and the immuno reactivity was assessed using the Vectastain Elite Kit/DAB (Vector Laboratories, NY). After counterstaining in Mayer´s hematoxyline, the sections were examined with Zeiss Axioscope and color video camera (ZVS-47E, Carl Zeiss Inc.).

Supplemental Table 1. Summary of *Helicobacter pylori* binding to glycosphingolipids

No.	Name	Sequence	1	2	Source
1.	**Lewis b**	**Fuc**a2Galb3GlcNAcb3Galb4Glcb1Cer 4 **Fuc**1	**+3**	**-**	Human small intestine
2.	**GM3**	**NeuAc**a3Galb4Glcb1Cer	-	**-**	Calf brain
3.	**GM1**	Galb3GalNAcb4Galb4Glcb1Cer 3 **NeuAc**a2	**-**	**-**	Calf brain
4.	**GD1a**	**NeuAc**a3Galb3GalNAcb4Galb4Glcb1Cer 3 **NeuAc**a2	**-**	**-**	Calf brain
5.	**GD1b**	Galb3GalNAcb4Galb4Glcb1Cer 3 **NeuAc**a8**NeuAc**a2	**-**	**-**	Calf brain
6.	**Sialyl-Lewis a**	**NeuAc**a3Galb3GlcNAcb3Galb4Glcb1Cer 4 **Fuc**1	**-**	**-**	Human gall bladder carcinoma
7.	**Sialyl-Lewis x**	**NeuAc**a3Galb4GlcNAcb3Galb4Glcb1Cer 3 **Fuc**1	**-**	**(+)**	Synthetic from Symbicom Ltd.
8.	**Sialyl-di-Lewis x**	**NeuAc**a3Galb4GlcNAcb3Galb4GlcNAcb3Galb4Glcb1Cer 3 3 **Fuc**a1 **Fuc**a1	**-**	**+3**	Human gall bladder carcinoma

1. Binding obtained with strain CCUG17875 **2**. Binding obtained with the *babA1A2* mutant strain. Binding is defined as follows: **+3** denotes a significant darkening on the autoradiogram when 1 pmol of substance was applied on the thin-layer plate; + denotes a darkening at 2 nmol; **(+)** an occasional darkening at 2 nmol; while – denotes no darkening even at 2 nmol.

Supplemental Table 2A. Gastric biopsies from 29 patients were scored for binding by the *babA1A2*-mutant and the parental strain 17875 in situ, and for several markers of inflammation.

Biopsy sample	17875-binding	*babA1A2*-binding	Lymphocyte (1)	PMN cell (2)	IH-total	IH-gastric pit	*H. pylori*	Hist. gastritis
TB 21	5(687)	1(16)	0	0	1	1	0	0,5
TB 16	4(459)	1(14)	0	0	1	1	0	0,5
TB 15	1(38)	0(0)	0	0	1	1	0	1
TB 14	5(712)	1(15)	0	1	3	2	0	0,5
TB 12	3(289)	4(48)	2	2	5	5	3	3
TB 9	5(633)	1(17)	1	1	1	1	0	0
HS+B+A	1(38)	0(0)	1	0	1	1	0	0,5
16519 A3	5(573)	1(13)	2	3	3	2	1	2
14167 A4	5(529)	1(15)	2	1	3	4	1	2
14814 A3	5(667)	1(13)	3	2	1	1	2	3
15393 A3	4(381)	4(51)	3	2	2	3	1	3
8900 A2	5(793)	1(16)	1	1	2	2	2	1
8956 A2	5(605)	2(23)	1	2	1	1	0,5	1
9220 A4	5(593)	2(19)	2	3	4	3	1	2
12486 A4	5(558)	0(0)	1	1	4	3	1	2
15143 A3	2(111)	2(21)	2	2	5	5	1	2
15606 A4	5(629)	3(33)	1	1	3	4	0	3
15981 A3	5(803)	0(0)	2	2	3	4	2	3
16849 A4	5(587)	1(13)	2	5	4	2	1	2
17961 A3	5(839)	3(37)	3	3	4	4	1	2
15754 A5	3(223)	3(29)	2	5	5	5	0,5	2
15754 B5	4(439)	4(43)	1	3	5	5	0,5	1
15187 A5	5(750)	3(38)	3	5	4	3	1	2
15187 B6	5(713)	1(14)	1	3	4	3	1	1
14690 B5	1(39)	4(51)	3	5	4	3	1	3
14322 A4	3(267)	1(15)	2	5	3	3	3	2
14322 B4	4(419)	3(28)	2	5	4	2	2	1
14238 A3	1(38)	4(47)	2	5	5	4	1	3
14238 B6	1(41)	3(38)	1	2	5	4	1	1

Supplemental Table 2B. Gastric biopsies from 6 *H. pylori* non-infected individuals were scored for binding in situ by the *babA1A2*-mutant and the parental strain 17875.

Biopsy sample	17875-binding	*babA1A2*-binding	*H. pylori* infection
No. 61	3(160)	1(12)	0
No. 62	4(485)	1(10)	0
No. 70	2(68)	0(7)	0
No. 64	4(463)	0(8)	0
D6	5(513)	0(8)	0
F73	2(96)	0(4)	0

Grading scale used for Tables 2A and 2B

CCUG17875-binding
Grading scale from 0 to 5, based on number of bacterial cells/gastric pit
Grade 0 = no bacteria, Grade 1 = 1-50 bacteria, Grade 2 = 50-150 bacteria
Grade 3 = 150-350 bacteria, Grade 4 = 350-500 bacteria, Grade 5 ≥500 bacteria

***babA1A2*-mutant binding**
Grading scale from 0 to 5, based on number of bacterial cells/ gastric pit
Grade 0 = no bacteria, Grade 1 = 10-15 bacteria, Grade 2 = 15-25 bacteria
Grade 3 = 25-40 bacteria, Grade 4 = 40-55 bacteria, Grade 5 ≥ 55 bacteria

Lymphocyte infiltration (1)
Grading scale from 0 to 3, based on both lymphocyte and plasmacell infiltration
Grade 0= normal, Grade 1 = low inflammation, Grade 2 = moderate inflammation, Grade 3 = heavy inflammation

PMN cell infiltration (2)
Grading scale from 0 to 5
Grade 0 = none, Grade 1 = rare PMN, only in lamina propria (LP), Grade 2 ≤ 1 intraepithelial (IE) PMN/high power field (hpf), i.e. 400X magnification, Grade 3 = 1-10 IE/hpf, Grade 4 ≥ 10 IE/hpf, Grade 5 = pit abscess

Immunohisto-staining by anti-sLex-mAb (IH)
Grading scale from 0 to 5
IH-total: IH-staining of whole tissue section
IH-gastric pit: IH-staining of gastric pit region

***H. pylori* in surface epithelium (Genta-stained)**
0.5 very few; 1 good colonization; 2 very good colonization; 3; heavy colonization

Histological gastritis
0.5-1 minimal; 2 superficial; 3: intense

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