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Redefinition of the Carbohydrate Binding Specificity of *Helicobacter pylori* BabA Adhesin*[S]

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Background: The BabA adhesin mediates binding of *Helicobacter pylori* to the gastric epithelium.
Results: Binding of BabA to blood group O and A determinants on type 4 core chains was demonstrated.
Conclusion: The BabA binds to blood group determinants on both type 1 and type 4 core chains.
Significance: Characterization of the binding specificities of BabA is important for understanding the interactions between *H. pylori* and target cells.

Certain *Helicobacter pylori* strains adhere to the human gastric epithelium using the blood group antigen-binding adhesin (BabA). All BabA-expressing *H. pylori* strains bind to the blood group O determinants on type 1 core chains, *i.e.* to the Lewis b antigen (Fucα2Galβ3(Fucα4)GlcNAc; Le[b]) and the H type 1 determinant (Fucα2Galβ3GlcNAc). Recently, BabA strains have been categorized into those recognizing only Le[b] and H type 1 determinants (designated specialist strains) and those that also bind to A and B type 1 determinants (designated generalist strains). Here, the structural requirements for carbohydrate recognition by generalist and specialist BabA were further explored by binding of these types of strains to a panel of different glycosphingolipids. Three glycosphingolipids recognized by both specialist and generalist BabA were isolated from the small intestine of a blood group O pig and characterized by mass spectrometry and proton NMR as H type 1 pentaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer), Globo H hexaglycosylceramide (Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer), and a mixture of three complex glycosphingolipids (Fucα2Galβ4GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer, Fucα2Galβ3GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer, and Fucα2Galβ4(Fucα3)GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer). In addition to the binding of both strains to the Globo H hexaglycosylceramide, *i.e.* a blood group O determinant on a type 4 core chain, the generalist strain bound to the Globo A heptaglycosylceramide (GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer), *i.e.* a blood group A determinant on a type 4 core chain. The binding of BabA to the two sets of isoreceptors is due to conformational similarities of the terminal disaccharides of H type 1 and Globo H and of the terminal trisaccharides of A type 1 and Globo A.

Attachment of microbes to cell surface receptors on the target tissue is considered an essential step in the initiation, establishment, and maintenance of infection. In recent years, a large number of studies have aimed at the identification of potential microbial host receptors, the majority of which appear to be glycoconjugates (1–3). Glycoconjugates exhibit a characteristic and specific pattern of expression, which is dependent on the animal species, age, individual, and cell type (4). Thus, the recognition of a specific carbohydrate receptor on the host cell surface determines at least in part the host, tissue, and age specificities of microbial infections.

Adherence of the gastric pathogen *Helicobacter pylori* to human gastric epithelial cells is required for prolonged persistence in the stomach. Initial studies of potential target cell receptors for *H. pylori* demonstrated the binding of certain strains of this bacterium to the Lewis b blood group antigen (Fucα2Galβ3(Fucα4)GlcNAc;[2] Le[b])[3] (5), and subsequently the *H. pylori* Le[b]-binding adhesin, blood group antigen-binding adhesin (BabA) was identified (6). *H. pylori* strains expressing BabA together with the vacuolating cytotoxin VacA and the cytotoxin-associated antigen CagA (triple positive strains) are associated with severe gastric diseases such as peptic ulcer and gastric adenocarcinoma (7, 8).

Subsequent studies demonstrated that the BabA adhesin has adapted to the fucosylated blood group antigens most prevalent in the local population (9). In Europe and the United States where blood group A, B, and O phenotypes all are common, the *H. pylori* strains (designated generalist strains) bind to blood group A, B, and O type 1 determinants. However, in populations such as the indigenous South American native population, which only has the blood group O phenotype, the *H. pylori*

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[S] This article contains supplemental Figs. S1 and S2.
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[2] The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (Chester, M. A. (1998) IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of glycolipids—recommendations 1997. *Eur. J. Biochem.* **257,** 293–298). It is assumed that Gal, Glc, GlcNAc, GalNAc, NeuAc, and NeuGc are of the D configuration; Fuc is of the L configuration; and all sugars are present in the pyranose form.
[3] The abbreviations used are: Le[b], Lewis b antigen; BabA, blood group antigen-binding adhesin; ESI, electrospray ionization; Hex, hexose; HexNAc, *N*-acetylhexosamine; Cer, ceramide; Le[y], Lewis y antigen; Le[a], Lewis a antigen; NeuGc, *N*-glycolylneuraminic acid.

strains (designated specialist strains) bind only to the blood group O type 1 determinants (Le^b and the H type 1). Thus, the carbohydrate binding site of BabA of generalist strains can accommodate an extension of the blood group O determinant with an α3-linked GalNAc or Gal (creating the blood group A and B determinants, respectively), whereas this extension is not tolerated by the BabA of specialist strains. Consequently, the BabA adhesins from these strains have differences in the architecture of their carbohydrate binding sites.

In the present study, the structural requirements for carbohydrate recognition by BabA of generalist and specialist *H. pylori* strains were further explored. Radiolabeled *H. pylori* strains were examined for binding to a panel of different glycosphingolipids from various sources separated on thin-layer plates, and glycosphingolipids recognized by wild type specialist and/or generalist *H. pylori*, but not by a deletion mutant strain lacking the BabA adhesin, were isolated and characterized by mass spectrometry and proton NMR. Comparative binding studies demonstrated that the BabA adhesin in addition to blood group determinants on type 1 core chains recognizes blood group O and A determinants on type 4 core chains with binding to Globo H (*i.e.* H type 4) by both strains and Globo A (*i.e.* A type 4) by the generalist strain. Inspection of minimum energy models revealed topographical similarities in the spatial orientation of the terminal disaccharide (Fucα2Galβ3) of the Globo H and H5 type 1 glycosphingolipids, accounting for the BabA cross-reactivity.

EXPERIMENTAL PROCEDURES

H. pylori Strains, Culture Conditions, and Labeling—The generalist *H. pylori* strain J99 and the construction of the J99/BabA− mutant *babA*::cam were described by Mahdavi *et al.* (10). The specialist *H. pylori* strain S831 was described (9).

For chromatogram binding experiments, the bacteria were grown in a microaerophilic atmosphere at 37 °C for 48 h on Brucella medium (Difco) containing 10% fetal calf serum (Harlan Sera-Lab, Loughborough, UK) inactivated at 56 °C and BBL IsoVitaleX Enrichment (BD Biosciences). The mutant strain J99/BabA− was cultured on the same medium supplemented with chloramphenicol (20 μg/ml). Bacteria were radiolabeled by the addition of 50 μCi [^{35}S]methionine (Amersham Biosciences) diluted in 0.5 ml of phosphate-buffered saline (PBS), pH 7.3 to the culture plates. After incubation for 12–72 h at 37 °C under microaerophilic conditions, the bacteria were harvested, centrifuged three times, and thereafter suspended to 1×10^8 cfu/ml in PBS. The specific activities of the suspensions were ~1 cpm/100 *H. pylori* organisms.

Chromatogram Binding Assays—Reference glycosphingolipids were isolated and characterized by mass spectrometry and proton NMR as described (11).

Thin-layer chromatography was performed on glass- or aluminum-backed silica gel 60 HPTLC plates (Merck). Mixtures of glycosphingolipids (40 μg) or pure compounds (40 ng–4 μg) were separated using chloroform/methanol/water (60:35:8 by volume) as the solvent system. Chemical detection was accomplished by anisaldehyde (12).

Binding of ^{35}S-labeled *H. pylori* to glycosphingolipids on thin-layer chromatograms was done as reported previously (13). Dried chromatograms were dipped for 1 min in diethyl ether/*n*-hexane (1:5 by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich). After drying, the chromatograms were soaked in PBS containing 2% bovine serum albumin (w/v), 0.1% NaN_3 (w/v), and 0.1% Tween 20 (by volume) for 2 h at room temperature. The chromatograms were subsequently covered with radiolabeled bacteria diluted in PBS ($2–5 \times 10^6$ cpm/ml). Incubation was done for 2 h at room temperature followed by repeated washings with PBS. The chromatograms were thereafter exposed to XAR-5 x-ray films (Eastman Kodak Co.) for 12 h.

Chromatogram binding assays with mouse monoclonal antibodies directed against the Globo H determinant (MBr1, Enzo Life Sciences), the Le^b determinant (BG-6/T218, Signet/Covance), the H type 1 determinant (17-206, Abcam), and the H type 2 determinant (A583, DakoCytomation Norden A/S) were done as described (13) using ^{125}I-labeled monoclonal anti-mouse antibodies (Z0259, DakoCytomation Norden A/S) for detection.

Isolation of H. pylori-binding Glycosphingolipids—Total acid and non-acid glycosphingolipid fractions were isolated by standard methods (11). Briefly, the material was lyophilized and then extracted in two steps in a Soxhlet apparatus with chloroform and methanol (2:1 and 1:9 by volume, respectively). The material obtained was subjected to mild alkaline hydrolysis and dialysis followed by separation on a silicic acid column. Acid and non-acid glycosphingolipid fractions were obtained by chromatography on a DEAE-cellulose column. To separate the non-acid glycolipids from alkali-stable phospholipids, this fraction was acetylated and separated on a second silicic acid column followed by deacetylation and dialysis. Final purifications were done by chromatographies on DEAE-cellulose and silicic acid columns.

The non-acid glycosphingolipid fractions were separated by repeated silicic acid chromatography, and final separation was achieved by HPLC or by chromatography on Iatrobead (Iatrobeads 6RS-8060, Iatron Laboratories, Tokyo, Japan) columns and elution with chloroform/methanol/water (65:25:4 by volume) followed by chloroform/methanol/water (60:35:8 by volume) and finally chloroform/methanol/water (40:40:12 by volume). Throughout the separation procedures, aliquots of the fractions obtained were analyzed by thin-layer chromatography, and fractions that were colored green by anisaldehyde were tested for binding of *H. pylori* using the chromatogram binding assay. The fractions were pooled according to the mobility on thin-layer chromatograms and their *H. pylori* binding activity.

Endoglycoceramidase Digestion and LC-ESI/MS—Endoglycoceramidase II from *Rhodococcus* spp. (14) (Takara Bio Europe S.A., Gennevilliers, France) was used for hydrolysis of glycosphingolipids. Briefly, 50 mg of glycosphingolipids were suspended in 100 ml of 0.05 M sodium acetate buffer, pH 5.0 containing 120 mg of sodium cholate and sonicated briefly. Thereafter, 1 milliunit of endoglycoceramidase II was added, and the mixture was incubated at 37 °C for 48 h. The reaction was stopped by addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-containing upper phase thus obtained was separated from deter-

gent on a Sep-Pak QMA cartridge (Waters, Milford, MA). The eluant containing the oligosaccharides was dried under nitrogen and under vacuum.

The glycosphingolipid-derived oligosaccharides were analyzed by LC/MS and MS/MS as described (15). In brief, the oligosaccharides were separated on a column (200 × 0.180 mm) packed in house with 5-mm porous graphite particles (Hypercarb, Thermo Scientific) and eluted with an acetonitrile gradient (A, 10 mM ammonium bicarbonate; B, 10 mM ammonium bicarbonate in 80% acetonitrile). The saccharides were analyzed in the negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA).

LC-ESI/MS and ESI/MS/MS of Native Glycosphingolipids—The glycosphingolipids (dissolved in methanol/acetonitrile, 75:25 by volume) were separated on a 200 × 0.150-mm column packed in house with 5-mm polyamine II particles (YMC Europe GmbH, Dinslaken, Germany) and eluted with a water gradient (A, 100% acetonitrile; B, 10 mM ammonium bicarbonate). Samples were analyzed on an LTQ linear quadrupole ion trap mass spectrometer by LC-ESI/MS at −3.5 kV. A full scan (*m*/*z* 500–1800; two microscans; maximum time, 100 ms; target value, 30,000) was performed followed by data-dependent MS^2 scans (two microscans; maximum time, 100 ms; target value, 10,000) with a normalized collision energy of 35%, an isolation window of 2.5 units, an activation q of 0.25, and an activation time of 30 ms.

Proton NMR Spectroscopy—^{1}H NMR spectra were acquired on a Varian 600-MHz spectrometer at 30 °C. Samples were dissolved in dimethyl sulfoxide/D_2O (98:2 by volume) after deuterium exchange. Two-dimensional double quantum-filtered correlated spectroscopy (COSY) spectra were recorded using the standard pulse sequence (16).

Molecular Modeling—Minimum energy models of different glycosphingolipids were constructed using the CHARMm force field within the Discovery Studio molecular modeling package (Accelrys, Inc., San Diego, CA) and literature values as starting points for the glycosidic torsion angles (17, 18).

RESULTS

Binding of H. pylori to Glycosphingolipid Mixtures—Screening for BabA-mediated binding of *H. pylori* was done by binding of the generalist *H. pylori* strain J99, the specialist strain S831, and the deletion mutant strain J99/BabA− to non-acid glycosphingolipid fractions from various sources to expose the bacteria to a large number of potentially binding-active carbohydrate structures. Thus, the binding of the bacteria to non-acid glycosphingolipid mixtures isolated from the small intestine of different species (human, rat, cat, and pig (19–23)), erythrocytes of different species (human, cat, rabbit, dog, horse, chicken, and sheep (24)), human cancers (lung, kidney, colon, liver, and gastric cancers (25)), and human stomach (26) was tested. Thereby, three glycosphingolipids recognized by both the generalist and specialist *H. pylori* strain were detected in the non-acid glycosphingolipid fraction from the small intestinal epithelium of a blood group O pig (Fig. 1, *B* and *C*, *lane 1*). The binding-active compounds migrated in the penta-, hexa-, and octa-/nonaglycosylceramide regions, respectively. No binding of the deletion mutant strain J99/BabA− to the porcine intestinal glycosphingolipids was obtained (data not shown), indicating that the binding of the wild type bacteria to these compounds was mediated by BabA.

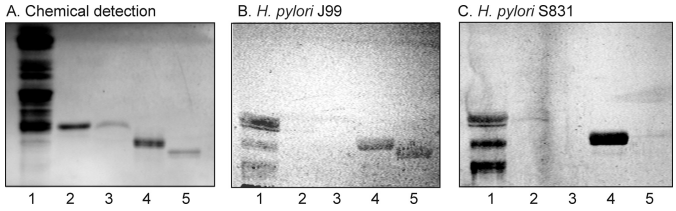

FIGURE 1. **Binding of a generalist and a specialist *H. pylori* strain to non-acid glycosphingolipids of the small intestinal epithelium of a blood group O pig.** The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8 by volume) as the solvent system. The chromatogram in *A* was stained with anisaldehyde. Duplicate chromatograms were incubated with the ^{35}S-labeled *H. pylori* generalist strain J99 (*B*) and the *H. pylori* specialist strain S831 (*C*) followed by autoradiography for 12 h as described under "Experimental Procedures." *Lane 1*, non-acid glycosphingolipids of the intestinal epithelium of a blood group O pig, 40 μg; *lane 2*, reference H type 2 pentaglycosylceramide (Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 μg; *lane 3*, reference Le^a pentaglycosylceramide (Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 4*, reference Le^b hexaglycosylceramide (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 4 μg; *lane 5*, reference B type 1 heptaglycosylceramide (Galα3(Fucα2)-Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg.

Isolation of the H. pylori-binding Glycosphingolipids from Porcine Intestine—A total non-acid fraction from blood group O porcine small intestinal epithelium (160 mg) was separated by repeated silica gel chromatography and Iatrobead column chromatography, and the subfractions obtained were tested for *H. pylori* binding activity. After pooling of binding-active fractions, three subfractions containing *H. pylori*-binding glycosphingolipids were obtained. One of these fraction (designated fraction P-I (0.2 mg)) migrated in the pentaglycosylceramide region, whereas the fraction designated fraction P-II (0.2 mg) migrated in the hexaglycosylceramide region (Fig. 2, *lanes 1* and *2*). LC-ESI/MS of the third fraction containing the slowest migrating *H. pylori*-binding compounds showed that this was a mixture of several glycosphingolipids. This fraction was therefore further separated on an Iatrobead column, and after pooling of the *H. pylori*-binding fractions, 0.3 mg of the slow migrating *H. pylori*-binding compound (designated fraction P-III) was obtained (Fig. 2, *lane 3*).

Characterization of the H. pylori-binding Fraction P-I from Porcine Intestine—LC-ESI/MS, proton NMR, and antibody binding demonstrated that fraction P-I was a mixture of the H type 1 pentaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer) and the B5 pentaglycosylceramide (Galα3-Galβ4GlcNAcβ3Galβ4Glcβ1Cer) (data not shown).

Characterization of the H. pylori-binding Fraction P-II from Porcine Intestine—Characterization of the BabA binding fraction P-II demonstrated the Globo H hexaglycosylceramide (Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer) as the major compound. This conclusion was based on the following properties. (i) ESI/MS of the native fraction P-II gave a major $[M - 2H^+]^{2-}$ ion at *m*/*z* 784, corresponding to a molecular ion at *m*/*z* 1568, demonstrating a glycosphingolipid with one Fuc, one HexNAc, and four Hex residues and phytosphingosine with hydroxy 16:0 fatty acid (data not shown). The series of C, Y, and Z ions obtained by MS^2 of the $[M - 2H^+]^{2-}$ ion at *m*/*z* 784

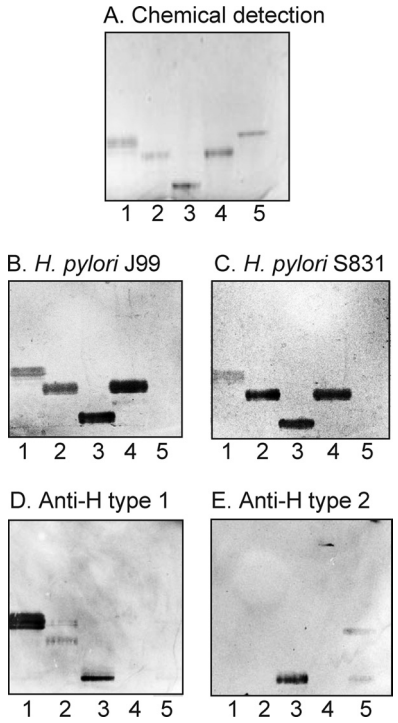

FIGURE 2. ***H. pylori*-binding glycosphingolipids isolated from the small intestinal epithelium of a blood group O pig.** The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8 by volume) as the solvent system. The chromatogram in *A* was stained with anisaldehyde. Duplicate chromatograms were incubated with the ^{35}S-labeled *H. pylori* generalist strain J99 (*B*), the *H. pylori* specialist strain S831 (*C*), the monoclonal anti-H type 1 antibody 17-206 (*D*), and the monoclonal anti-H type 2 antibody 92FR-A2 (*E*) followed by autoradiography for 12 h as described under "Experimental Procedures." *Lane 1*, fraction P-I isolated from pig intestine, 2 μg; *lane 2*, fraction P-II from pig intestine, 2 μg; *lane 3*, fraction P-III from pig intestine, 2 μg; *lane 4*, reference Le^b hexaglycosylceramide (Fucα2-Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 5*, reference H type 2 pentaglycosylceramide (Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 μg.

demonstrated a Fuc-Hex-HexNAc-Hex-Hex-Hex sequence (supplemental Fig. S1).

(ii) LC-ESI/MS of oligosaccharides gives the resolution of isomeric saccharides, and the carbohydrate sequence can be deduced from series of C type fragment ions obtained by MS^2 (15). In addition, diagnostic cross-ring ^{0,2}A type fragment ions are present in MS^2 spectra of oligosaccharides with a Hex or HexNAc substituted at C-4 and thus allow differentiation of linkage positions (15, 27, 28).

LC-ESI/MS of the oligosaccharides obtained by hydrolysis of fraction P-II with *Rhodococcus* endoglycoceramidase II gave two late eluting molecular ions (Fig. 3, *A*–*C*). These ions were found at *m*/*z* 852 (retention time, 26.8–27.2 min) and at *m*/*z* 1014 (retention time, 25.2–25.7 min) and demonstrated one oligosaccharide with one Fuc, one HexNAc and three Hex residues and one oligosaccharide with one Fuc, one HexNAc, and four Hex residues, respectively.

The MS^2 spectrum of the molecular ion at *m*/*z* 852 (Fig. 3*D*) had a C type fragment ion series (C_2 at *m*/*z* 325, C_3 at *m*/*z* 528, and C_4 at *m*/*z* 690), demonstrating a Fuc-Hex-HexNAc-Hex-Hex sequence. The features of this MS^2 spectrum were very similar to the MS^2 spectrum of reference H type 1 pentaglycosylceramide (15).

MS^2 of the molecular ion at *m*/*z* 1014 (Fig. 3*E*) also gave a series of C type fragment ions with C_2 at *m*/*z* 325, C_3 at *m*/*z* 528, and C_4 at *m*/*z* 690 along with a C_5 ion at *m*/*z* 852, identifying a Fuc-Hex-HexNAc-Hex-Hex-Hex sequence. The $^{0,2}A_5$ fragment ion at *m*/*z* 792 and the $^{0,2}A_6$ fragment ion at *m*/*z* 954 indicated that the two hexoses at the reducing end were substituted at C-4, *i.e.* a Fuc-Hex-HexNAc-Hex-4Hex-4Hex sequence.

(iii) The anomeric region of the proton NMR spectrum of fraction P-II (Fig. 3*F*) revealed a single dominating species with six carbohydrate residues that is identical to the previously published Globo H glycosphingolipid (29) as evidenced by signals at 4.949 (Fucα2), 4.802 (Galα4), 4.468 (GalNAcβ3), 4.456 (Galβ3), 4.247 (Galβ4), and 4.208 ppm (Glcβ1), thus yielding the sequence Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer in accordance with the mass spectrometry data above.

Thus, by mass spectrometry and proton NMR, the BabA-binding hexaglycosylceramide of blood group O pig intestine was identified as the Globo H glycosphingolipid. In the base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained by hydrolysis of fraction P-II with *Rhodococcus* endoglycoceramidase (Fig. 3*A*), the major molecular ion was found at *m*/*z* 852, corresponding to the H type 1 pentaglycosylceramide. Still, proton NMR demonstrated that fraction P-II was a relatively pure Globo H glycosphingolipid. This discrepancy is due to the restricted hydrolytic capacity of the *Rhodococcus* endoglycoceraminidase II, which has a relative resistance of hydrolysis for globo series glycosphingolipids (14, 30). The ideal enzyme would have been the ceramide glycanase from *Macrobdella decora* that has a more universal hydrolytic activity toward glycosphingolipids (31). However, the *M. decora* enzyme is no longer available commercially.

Characterization of the Slow Migrating H. pylori-binding Fraction P-III from Porcine Intestine—Antibody binding, mass spectrometry, and proton NMR demonstrated that fraction P-III was a mixture of two branched decaglycosylceramides with terminal H type 1 epitopes (Fucα2Galβ3GlcNAcβ6-(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer and Fucα2Galβ4GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer) and a related undecaglycosylceramide with a Fucα3 substitution of the GlcNAc of the 6-branch, yielding an Le^y determinant (Fucα2Galβ4(Fucα3)GlcNAcβ6-(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer). This conclusion is based on the following observations. (i) The glycosphingolipid fraction P-III was stained by both the anti-H type 1 antibody and the anti-H type 2 antibody (Fig. 2, *D* and *E*, *lane 3*).

(ii) ESI/MS of the native fraction P-III gave a major $[M - 2H^+]^{2-}$ ion at *m*/*z* 1132, corresponding to a molecular ion at *m*/*z* 2264, indicating a decasaccharide with two Fuc, three HexNAc, and five Hex residues combined with sphingosine and non-hydroxy 16:0 fatty acid (data not shown). In addition, there was an $[M - 2H^+]^{2-}$ ion at *m*/*z* 1205, corresponding to a molecular ion at *m*/*z* 2410, suggesting an undecasaccharide with three Fuc, three HexNAc, and five Hex residues combined with sphingosine and non-hydroxy 16:0 fatty acid.

(iii) LC-ESI/MS of the oligosaccharides obtained by hydrolysis of fraction P-III with *Rhodococcus* endoglycoceramidase II had two $[M - 2H^+]^{2-}$ ions at *m*/*z* 864, corresponding to molec-

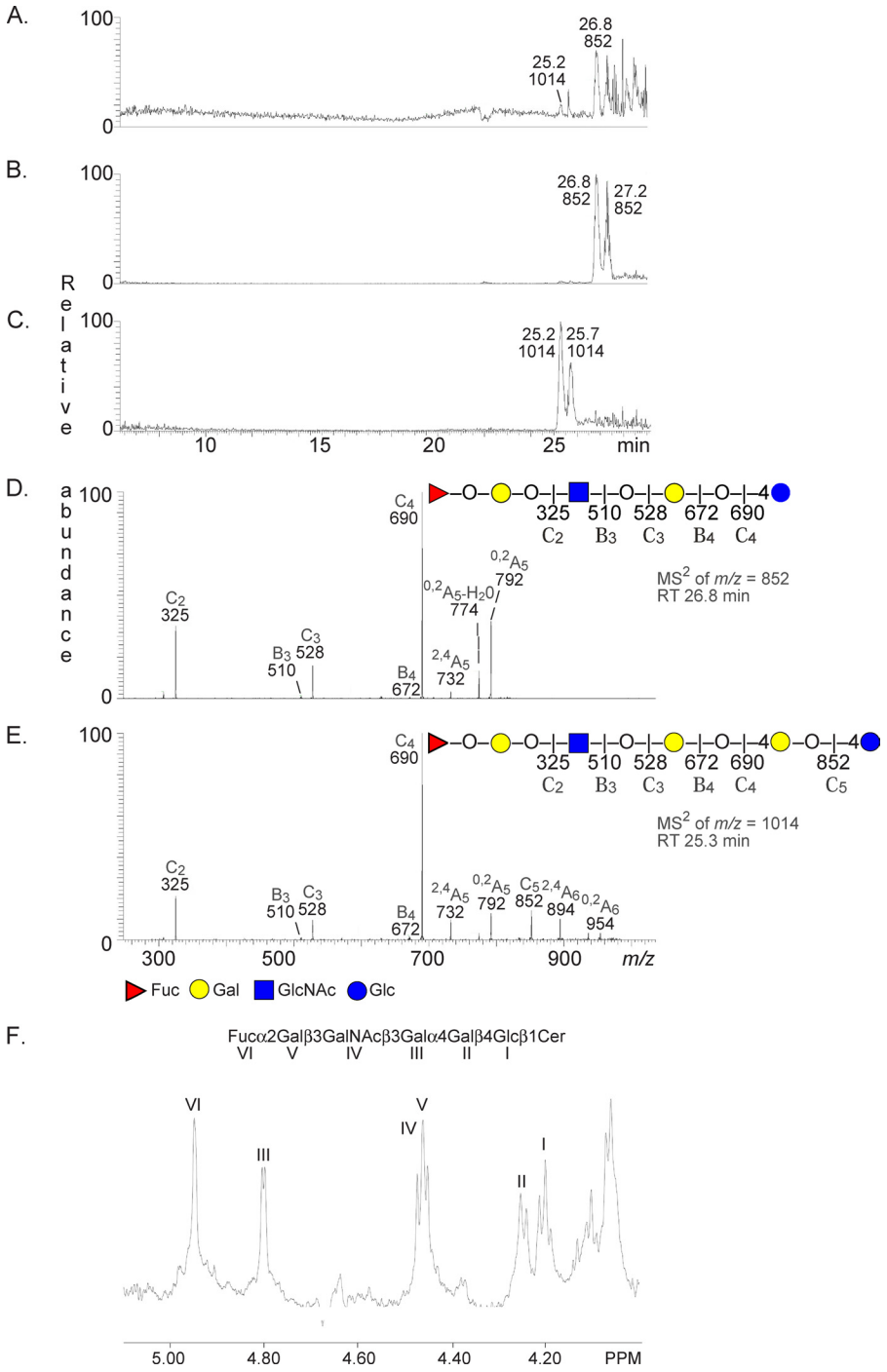

FIGURE 3. **Characterization of the *H. pylori* BabA-binding fraction P-II from the small intestinal epithelium of a blood group O pig.** *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained by digestion of the *H. pylori* BabA-binding fraction P-II with *Rhodococcus* endoglycoceramidase II. *B*, mass chromatogram of *m/z* 852. *C*, mass chromatogram of *m/z* 1014. *D*, MS^2 spectrum of the $[M - H^+]^-$ ion at *m/z* 852 (retention time (*RT*), 26.8 min). The interpretation formula shows the deduced oligosaccharide sequence. *E*, MS^2 spectrum of the $[M - H^+]^-$ ion at *m/z* 1014 (retention time, 25.3 min). The interpretation formula shows the deduced oligosaccharide sequence. *F*, anomeric region of the 600-MHz proton NMR spectrum of fraction P-II (30 °C). The sample was dissolved in dimethyl sulfoxide/D_2O (98:2 by volume) after deuterium exchange.

ular ions at *m/z* 1728, demonstrating two decasaccharides, both with two Fuc, three HexNAc, and five Hex residues (supplemental Fig. S2). The minor $[M - 2H^+]^{2-}$ ion eluted at 23.4–24.5 min, and the major $[M - 2H^+]^{2-}$ ion eluted at 25.8–26.1 min. The MS^2 spectra of the minor and major $[M - 2H^+]^{2-}$ ions both had weak lower mass regions, but in both cases, a terminal Fuc-Hex-HexNAc sequence was indicated by C_2 ions at *m/z* 325 and/or C_3 ions at *m/z* 528 or B_3 ions at *m/z* 510 (Fig. 4, *A* and *B*). In addition, there were intense C type ions at *m/z* 1201, 1404, and 1566.

MS^3 of the ion at *m/z* 1201 at retention time 23.4 min gave a C_3 ion at *m/z* 528, again demonstrating a terminal Fuc-Hex-HexNAc sequence (Fig. 4*C*). In contrast, the MS^3 spectrum of the ion at *m/z* 1201 at retention time 26.3 min was dominated

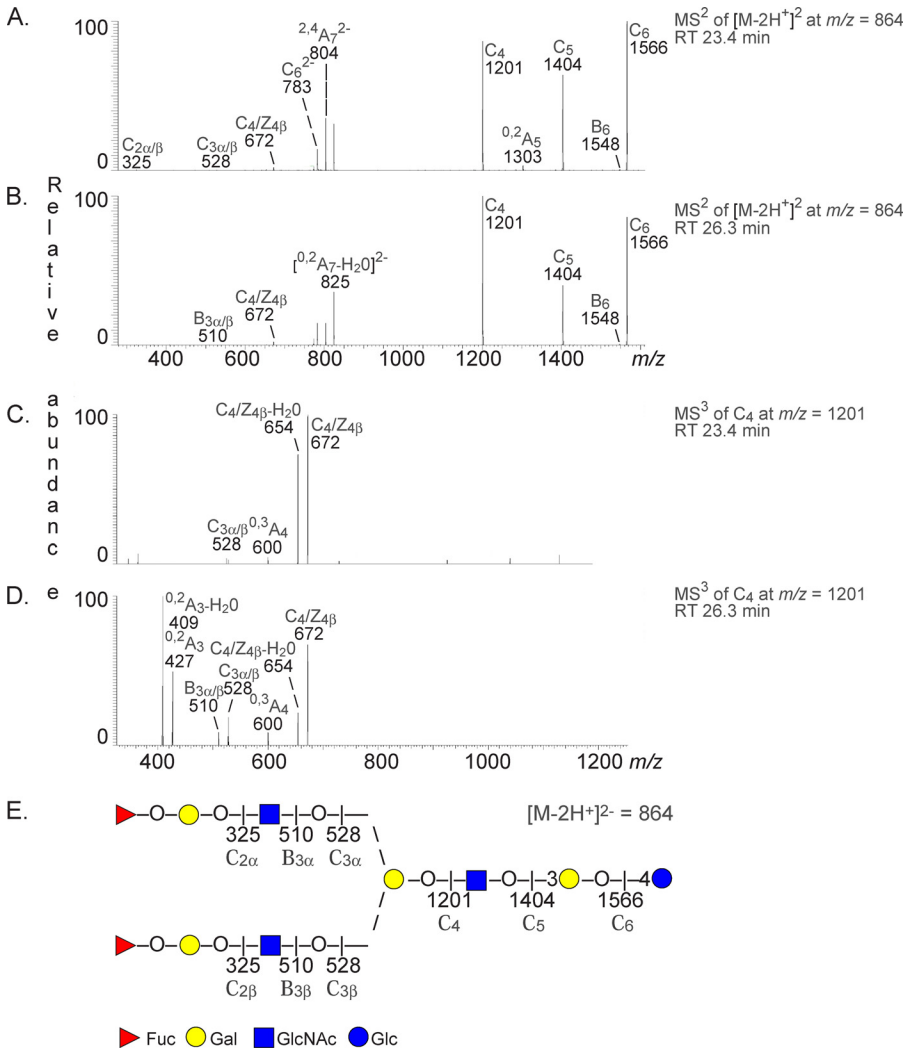

FIGURE 4. **LC-ESI/MS of the decasaccharides obtained by hydrolysis of *H. pylori*-binding fraction P-III with *Rhodococcus* endoglycoceramidase II.** *A*, MS^2 spectrum of the $[M - 2H^+]^{2-}$ ion at *m/z* 864 (retention time (*RT*), 23.4 min). *B*, MS^2 spectrum of the $[M - 2H^+]^{2-}$ ion at *m/z* 864 (retention time, 26.3 min). *C*, MS^3 spectrum of the ion at *m/z* 1201 (retention time, 23.4 min). *D*, MS^3 spectrum of the ion at *m/z* 1201 (retention time, 26.3 min). *E*, interpretation formula showing the deduced oligosaccharide sequence.

by an intense $^{0,2}A_3$ ion at *m/z* 427 and a $^{0,2}A_3 - H_2O$ ion at *m/z* 409, which together with the C_3 ion at *m/z* 528 identified a terminal Fuc-Hex-HexNAc sequence with 4-substitution of the HexNAc, *i.e.* a type 2 core chain (Fig. 4*D*) (15, 27, 28).

Both MS^3 spectra (Fig. 4, *C* and *D*) had $C_4/Z_{4\beta}$ ions at *m/z* 672. These ions are obtained by double glycosidic cleavage at the 3-linked bond of the branched Hex residue and thus comprise the 6-linked carbohydrate chain and the core branching Hex residue (32). The $^{0,3}A_4$ ions at *m/z* 600 obtained by cross-ring cleavages present in both MS^3 spectra further confirm the Fuc-Hex-HexNAc sequence on the 6-branch (32).

Thus, these MS^2 and MS^3 spectral features suggested that fraction P-III contained two branched decasaccharides, *i.e.* two Fuc-Hex-HexNAc-(Fuc-Hex-HexNAc-)Hex-HexNAc-Hex-Hex saccharides. The terminal Fuc-Hex-HexNAc sequences of the minor compound had type 1 core chains, whereas the terminal Fuc-Hex-HexNAc sequences of the major compound had type 2 core chain on at least one branch.

The LC-ESI/MS base peak chromatogram of the oligosaccharides from fraction P-III (supplemental Fig. S2) also had an $[M - 2H^+]^{2-}$ ion at *m/z* 937, corresponding to a molecular ion at *m/z* 1874, indicating an undecasaccharide with three Fuc, three HexNAc, and five Hex residues. In addition, the MS^2 and MS^3 spectra obtained had weak lower mass regions (Fig. 5). There was a C_2 ion at *m/z* 325 and a B_3 ion at *m/z* 510, indicating a terminal Fuc-Hex-HexNAc sequence. In addition, the B_3 ion at *m/z* 656 demonstrated a terminal Fuc-Hex-(Fuc-)HexNAc sequence. This was confirmed by the $C_4/Z_{4\beta}$ ion at *m/z* 818, comprising the 6-linked carbohydrate chain and the core branching Hex residue, and the $^{0,3}A_4$ cross-ring cleavage ion at *m/z* 746. Furthermore, both the $C_4/Z_{4\beta}$ ion and the $^{0,3}A_4$ ion demonstrated that the Fuc-Hex-(Fuc-)HexNAc sequence was carried by the 6-branch (32). The spectra also had a series of prominent C type fragment ions (C_4 at *m/z* 1347, C_5 at *m/z* 1550, and C_6 at *m/z* 1712). Taken all together, MS^2 and MS^3 indicated a branched undecasaccharide (Fuc-Hex-(Fuc-)HexNAc-(Fuc-Hex-HexNAc-)-Hex-HexNAc-Hex-Hex with a Fuc-Hex-(Fuc-)HexNAc sequence on the 6-branch and an H type 1 epitope on the 3-branch.

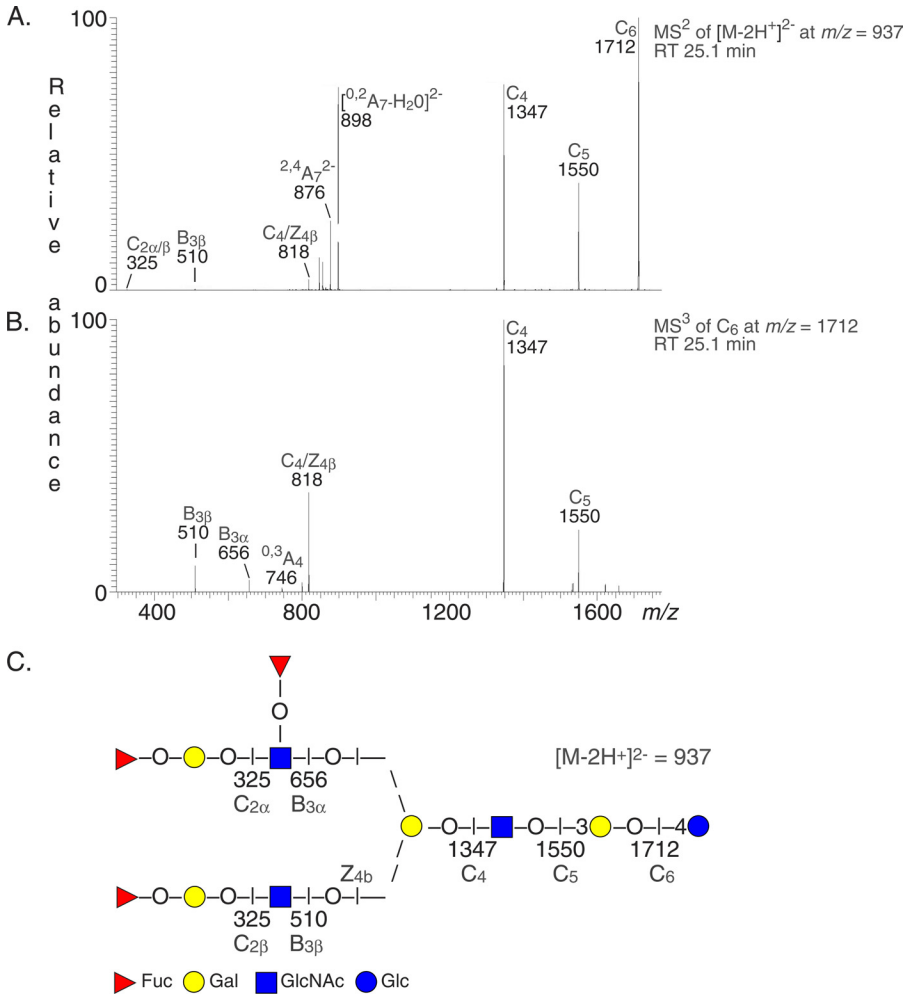

FIGURE 5. **LC-ESI/MS of the undecasaccharide obtained by hydrolysis of *H. pylori*-binding fraction P-III with *Rhodococcus* endoglycoceramidase II.** *A*, MS^2 spectrum of the $[M - 2H^+]^{2-}$ ion at *m/z* 937 (retention time (*RT*), 25.1 min). *B*, MS^3 spectrum of the ion at *m/z* 1712 (retention time, 25.1 min). *C*, interpretation formula showing the deduced oligosaccharide sequence.

(iv) The anomeric region of the proton NMR spectrum of fraction P-III is shown in Fig. 6. Fraction P-III contains two decaglycosylceramides (Fucα2Galβ4GlcNAcβ6(Fucα2-Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer and Fucα2Galβ3GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer) that have been isolated previously from rat (33) and pig intestine (23) and characterized in detail by NMR (using DMSO/D_2O (98:2) as solvent). In fraction P-III, the glycosphingolipid with mixed type 1/type 2 branches (Fucα2Galβ4GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer) is the major compound as evidenced by the relative intensities of the Fucα2 signals. The chemical shift data are summarized in Table 1. In addition, a novel glycosphingolipid structure with an Le^y determinant on the 6-branch and an H type 1 determinant on the 3-branch (Fucα2-Galβ4(Fucα3)GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3-GlcNAcβ3Galβ4Glcβ1Cer) could be characterized as shown in Fig. 6 and Table 1.

Comparative Glycosphingolipid Binding Assays—Thereafter, the binding of the specialist *H. pylori* strain S831 and the generalist strain J99 to a number of reference glycosphingolipids was evaluated. The results are summarized in Table 2. When using this set of reference glycosphingolipids, only the Le^b hexaglycosylceramide was recognized by the specialist strain S831 (Fig. 7*C*, *lane 1*), whereas the generalist *H. pylori* strain J99 in addition to the Le^b hexaglycosylceramide bound to the A type 1 hexaglycosylceramide (GalNAcα3(Fucα2)Galβ3-GlcNAcβ3Galβ4Glcβ1Cer; Fig. 7*B*, *lane 4*), the B type 1 hexaglycosylceramide (Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4-Glcβ1Cer; Table 2, Number 9), the A type 1 heptaglycosylceramide (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4-Glcβ1Cer; Fig. 7*B*, *lane 3*), the B type 1 heptaglycosylceramide (Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer; Fig. 7*B*, *lane 2*), the A type 1 octaglycosylceramide (GalNAcα3-(Fucα2)Galβ3GlcNAcβ3Galβ3GlcNAcβ3Galβ4Glcβ1Cer; Table 2, Number 17), and the repetitive A type 1 nonaglycosylceramide (GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ3GlcNAcβ3-Galβ4Glcβ1Cer; Fig. 7*B*, *lane 6*). Furthermore, the chromatogram binding assay revealed that the A type 4 heptaglycosylceramide (Globo A; GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glc-β1Cer; Fig. 7*B*, *lane 5*) was also recognized by the generalist strain.

However, no type 2 core counterparts of these compounds were recognized such as *e.g.* the H type 2 pentaglycosylceramide (Fig. 1, *lane 2*; Table 2, Number 5), the Le^y hexaglycosylceramide (Number 8), the A type 2 hexaglycosylceramide (Number 12), the B type 2 hexaglycosylceramide (Number 10), the A

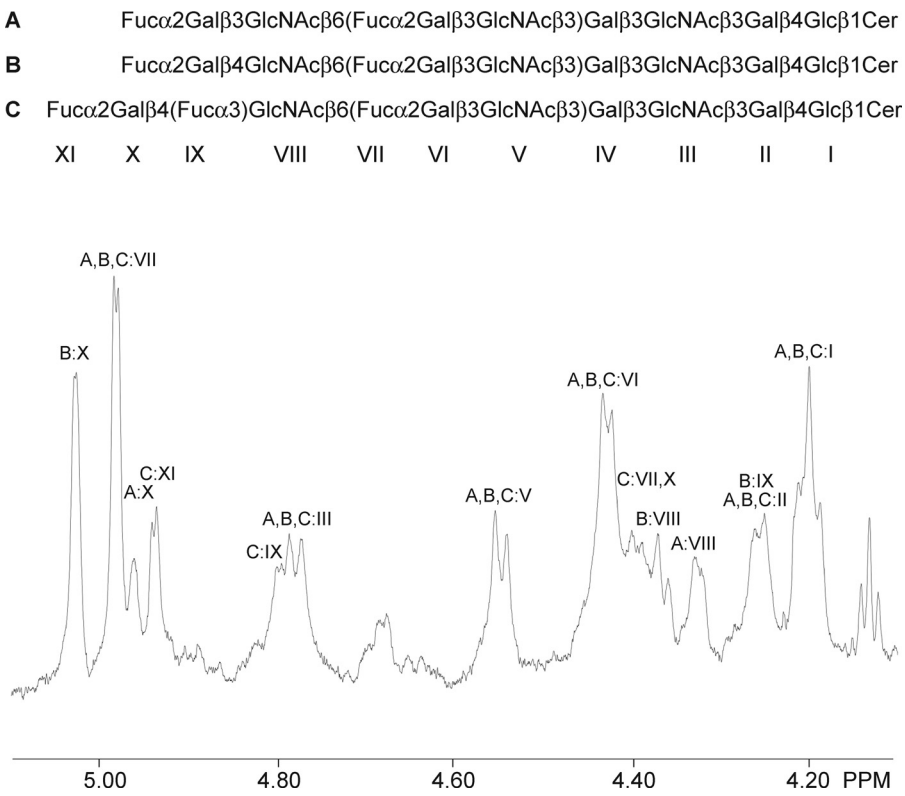

FIGURE 6. **Anomeric region of the 600-MHz proton NMR spectrum of the *H. pylori*-binding fraction P-III from porcine small intestinal epithelium (30 °C).** The sample was dissolved in dimethyl sulfoxide/D_2O (98:2 by volume) after deuterium exchange.

TABLE 1

Chemical shifts (ppm) of anomeric resonances of glycosphingolipids in fraction P-III from porcine small intestine dissolved in dimethyl sulfoxide/D_2O (98:2 by volume) identified by 600-MHz NMR at 30 °C

Structure	XI	X	IX	VIII	VII	VI	V	IV	III	II	I	
A		Fucα2	Galβ3	GlcNAcβ6	(Fucα2)	Galβ3	(GlcNAcβ3)	Galβ3	GlcNAβ3	Galβ4	Glcβ1	Cer
		4.962	4.41	4.36	4.982	4.427	4.547	4.20	4.779	4.26	4.19	
B		Fucα2	Galβ4	GlcNAcβ6	(Fucα2)	Galβ3	(GlcNAcβ3)	Galβ3	GlcNAβ3	Galβ4	Glcβ1	Cer
		5.028	4.33	4.33	4.982	4.427	4.547	4.20	4.779	4.26	4.19	
C	(Fucα2)	Galβ4	(Fucα3)	GlcNAcβ6)	(Fucα2)	Galβ3	(GlcNAcβ3)	Galβ3	GlcNAβ3	Galβ4	Glcβ1	Cer
	4.939	4.39	4.798	4.41	4.982	4.427	4.547	4.20	4.779	4.26	4.19	
D						Fucα2	Galβ4	(Fucα3)	GlcNAβ3	Galβ4	Glcβ1	Cer[a]
						4.962	4.398	4.852	4.680	4.261	4.218	

[a] For comparative purposes the chemical shift values are given for the Le^y hexaglycosylceramide from human erythrocytes characterized by Clausen *et al.* (41) at 30 °C. It is noteworthy that although the Fucα2 signals in the Le^y determinants are only separated by 0.020 ppm for structures C and D the corresponding Fucα3 signals are separated by as much as 0.054 ppm due to the fact that the determinant is located on a 6-branch. Le^y determinants on a 6-branch have not been described previously. This conclusion is confirmed, however, by examining the H5/H6 correlations in the double quantum-filtered COSY spectrum, which clearly reveals cross-peaks at 4.00/1.08 and 4.68/1.03 ppm originating from the Fucα2 and Fucα3 residues, respectively, of a Le^y determinant.

type 2 heptaglycosylceramide (Fig. 8*B*, *lane 7*; Number 15), and the A type 2 nonaglycosylceramide (Number 19). Furthermore, the A tetraglycosylceramide (Number 1) and the A type 3 nonaglycosylceramide (Number 20) were also non-binding.

When the generalist and specialist *H. pylori* strains were compared with respect to their ability to bind to dilutions of the binding-active glycosphingolipids on thin-layer chromatograms, the Le^b hexaglycosylceramide was the preferred ligand of both strains, and two strains bound to this compound with similar detection limits (Fig. 8, *lanes 1–3*). In addition, the generalist strain J99 bound to the GalNAcα3-substituted Le^b (*i.e.* the A type 1 heptaglycosylceramide), the Globo A heptaglycosylceramide, and the nonaglycosylceramide with repetitive type 1 blood group A determinants in all cases with detection limits at ~40 ng (Fig. 8*A*).

Molecular Modeling—Inspection of the minimum energy models of the H type 1 pentaglycosylceramide and the Globo H hexaglycosylceramide revealed a substantial topographical similarity, which makes it reasonable that these two compounds may be accommodated within the same carbohydrate binding site of BabA (Fig. 9). In contrast, the terminal disaccharide of the non-binding H type 2 pentaglycosylceramide (*right*) is rotated relative to the same disaccharide in the H type 1 pentaglycosylceramide (*left*) and the Globo H hexaglycosylceramide (*center*) by ~90°, explaining why this compound is non-binding.

Binding of Anti-Globo H to Glycosphingolipids from Human Stomach—Having established that *H. pylori* recognizes the Globo H glycosphingolipid, we next examined whether this glycosphingolipid is present in the target tissue of *H. pylori* by binding of monoclonal antibodies directed against the Globo H determinant to non-acid glycosphingolipid fractions from human stomach. Thereby, binding in the hexaglycosylceramide region was observed in the non-acid fractions from the stomach of the two individuals tested (Fig. 10*B*, *lanes 1* and *2*). Both human stomach

TABLE 2

Comparison of glycosphingolipid binding preferences of a generalist H. pylori strain (J99), a specialist H. pylori strain (S831), and a BabA deletion mutant H. pylori strain (J99/BabA−)

No.	Abbreviation	Structure	*H. pylori* J99	*H. pylori* S831	*H. pylori* J99/BabA−	Source (Ref.)
1	A-4	GalNAcα3(Fucα2)Galβ4Glcβ1Cer				Rat intestine (20)
2	Le^a-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−[a]	−	−	Human intestine (42)
3	Le^x-5	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Dog intestine (42)
4	H5 type 1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	+	−	Porcine intestine (22)
5	H5 type 2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Human erythrocytes (43)
6	H6 type 4 (Globo H)	Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer	+	+	−	Porcine intestine[b]
7	Le^b-6	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	+	+	−	Human intestine (42)
8	Le^y-6	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Dog intestine (42)
9	B6 type 1	Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	−	−	Human intestine (19)
10	B6 type 2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Human erythrocytes (44)
11	A6 type 1	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	−	−	Human intestine (19)
12	A6 type 2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Human erythrocytes (45)
13	B7 type 1	Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	+	−	−	Human intestine (19)
14	A7 type 1	GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	+	−	−	Human intestine (19)
15	A7 type 2	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Human erythrocytes (45)
16	A7 type 4 (Globo A)	GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer	+	−	−	Porcine intestine (22)
17	A8 type 1	GalNAcα3(Fucα2)Galβ3GlcNAcβGalβ3GlcNAcβ3Galβ4Glcβ1Cer	+	−	−	Porcine intestine (22)
18	A9 type 1	GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	−	−	Porcine intestine (22)
19	A9 type 2	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Cat intestine (21)
20	A9 type 3	GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Human erythrocytes (46)
21	Dimeric Le^a	Galβ3(Fucα4)GlcNAcβ3Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	Human intestine[c]
22	Dimeric Le^x	Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	[d]
23		Fucα2Galβ3GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+[e]	+	−	Pig intestine[b]
24		Fucα2Galβ4GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+[e]	+	−	Pig intestine[b]
25		Fucα2Galβ4(Fucα3)GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+[e]	+	−	Pig intestine[b]
26	NeuGc-nL6	NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	+	Rabbit thymus (47)

[a] Binding is defined as follows: + denotes a binding when 1 μg of the glycosphingolipid was applied on the thin-layer chromatogram, whereas − denotes no binding even at 4 μg.
[b] Present study.
[c] J. Benktander, J. Ångström, H. Karlsson, M. Lebens, and S. Teneberg, submitted manuscript.
[d] Glycosphingolipid Number 22 was prepared from sialyl-dimeric Le^x (10) by mild acid hydrolysis.
[e] *H. pylori* binding to glycosphingolipids Numbers 23–25 was determined using a mixture of the three compounds.

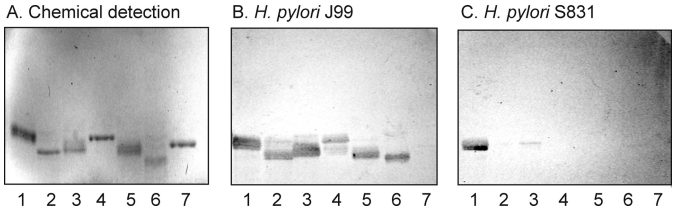

FIGURE 7. **Comparison of binding of a generalist and a specialist *H. pylori* strain to reference glycosphingolipids.** The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8 by volume) as the solvent system. The chromatogram in *A* was stained with anisaldehyde. Duplicate chromatograms were incubated with the ^{35}S-labeled *H. pylori* generalist strain J99 (*B*) and the *H. pylori* specialist strain S831 (*C*) followed by autoradiography for 12 h as described under "Experimental Procedures." *Lane 1*, Le^b hexaglycosylceramide (Fucα2Galβ3-(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 2*, B type 1 heptaglycosylceramide (Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 3*, A type 1 heptaglycosylceramide (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 4*, A type 1 hexaglycosylceramide (GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 5*, Globo A/A type 4 heptaglycosylceramide (GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer), 2 μg; *lane 6*, A nonaglycosylceramide (GalNAcα3-(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 7*, A type 2 heptaglycosylceramide (GalNAcα3(Fucα2)Galβ4(Fucα3)-GlcNAcβ3Galβ4Glcβ1Cer), 2 μg.

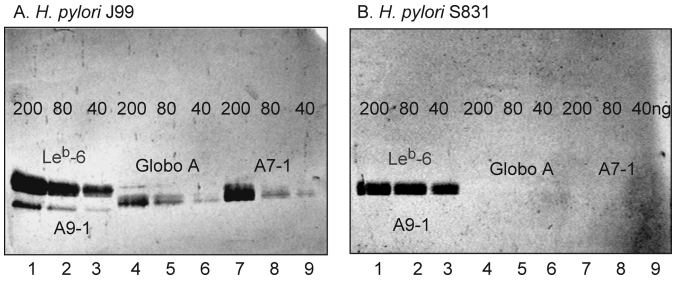

FIGURE 8. **Comparison of binding of a generalist and a specialist *H. pylori* strain to dilutions of pure glycosphingolipids on thin-layer chromatograms.** The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8 by volume) as the solvent system. The chromatograms were incubated with the ^{35}S-labeled *H. pylori* generalist strain J99 (*A*) and the *H. pylori* specialist strain S831 (*B*) followed by autoradiography for 12 h as described under "Experimental Procedures." *Lane 1*, Le^b hexaglycosylceramide (*Le^b-6*; Fucα2Galβ3-(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 200 ng, and A nonaglycosylceramide (*A9-1*; GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 200 ng; *lane 2*, Le^b hexaglycosylceramide, 80 ng, and A nonaglycosylceramide, 80 ng; *lane 3*, Le^b hexaglycosylceramide, 40 ng, and A nonaglycosylceramide, 40 ng; *lane 4*, Globo A/A type 4 heptaglycosylceramide (*Globo A*; GalNAcα3(Fucα2)-Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer), 200 ng; *lane 5*, Globo A/A type 4 heptaglycosylceramide, 80 ng; *lane 6*, Globo A/A type 4 heptaglycosylceramide, 40 ng; *lane 7*, A type 1 heptaglycosylceramide (*A7-1*; GalNAcα3(Fucα2)-Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 200 ng; *lane 8*, A type 1 heptaglycosylceramide, 80 ng; *lane 9*, A type 1 heptaglycosylceramide, 40 ng.

samples also contained the Le^b hexaglycosylceramide as indicated by the binding of the anti-Le^b antibody (Fig. 10*C*). The anti-H type 1 antibody cross-reacted with the Globo H glycosphingolipid to some extent. However, no binding of the anti-H type 1 antibody to the non-acid glycosphingolipid fractions from human stomach was obtained, although it bound intensely to the pentaglycosylceramide and to the slow migrating glycosphingolipid of blood group O pig intestine (Fig. 10*D*).

DISCUSSION

The binding of microbes to host target cells is crucial to the delivery of virulence factors, and in the case of *H. pylori*, it was recently shown that BabA-mediated binding of the bacteria to Le^b on the epithelium leads to an increased type IV secretion

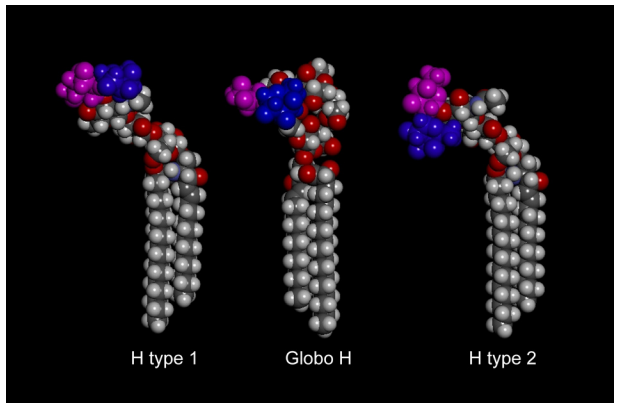

FIGURE 9. **Minimum energy models of the H type 1 pentaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer) (*left*), the Globo H hexaglycosylceramide (Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer) (*center*), and the H type 2 pentaglycosylceramide (Fucα2Galβ-4GlcNAcβ3Galβ4Glcβ1Cer) (*right*).** The terminal disaccharides are colored *blue* (Fucα2) and *purple* (Galβ3/4). The terminal part of the BabA-binding H type 1 and Globo H glycosphingolipids can be aligned by assuming different Glcβ1Cer torsion angles, whereas the H type 2 terminal disaccharide is rotated ~90° in comparison, rendering this glycosphingolipid non-binding with respect to BabA.

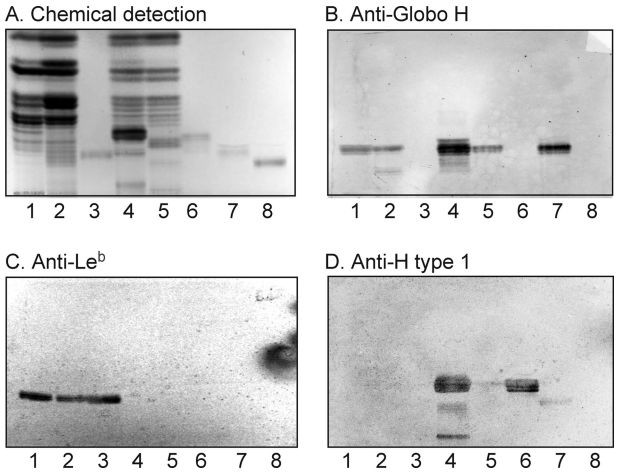

FIGURE 10. **Binding of monoclonal antibodies to human gastric glycosphingolipids.** Thin-layer chromatogram after detection with anisaldehyde (*A*) and autoradiograms obtained by binding of the monoclonal anti-Globo H antibody MBr1 (*B*), the monoclonal anti-Le^b antibody BG-6/T218 (*C*), and the monoclonal anti-H type 1 antibody 17-206 (*D*) are shown. The chromatograms were eluted with chloroform/methanol/water (60:35:8 by volume), and the binding assays were done as described under "Experimental Procedures" followed by autoradiography for 12 h. *Lane 1*, non-acid glycosphingolipids of human stomach (Individual I; blood group O), 40 μg; *lane 2*, non-acid glycosphingolipids of human stomach (Individual II; blood group A), 40 μg; *lane 3*, reference Le^b hexaglycosylceramide (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 4*, non-acid glycosphingolipids of the intestinal epithelium of a blood group O pig, 40 μg; *lane 5*, non-acid glycosphingolipids of the intestinal epithelium of a blood group A pig, 40 μg; *lane 6*, reference H type 1 pentaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; *lane 7*, reference Globo H hexaglycosylceramide (Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer), 1 μg; *lane 8*, reference Globo A heptaglycosylceramide (GalNAcα3(Fucα2)Galβ3GalNAc-β3Galα4Galβ4Glcβ1Cer), 4 μg.

system activity, resulting in the production of proinflammatory cytokines and precancer-related factors (34).

The initial observation that the fucosylated blood group antigens H type 1 and Le^b are mediators of *H. pylori* adhesion to human gastric epithelial cells (5) was followed by a division of BabA-producing *H. pylori* strains into specialist and generalist strains, depending on their mode of binding to Le^b and related

carbohydrate sequences (9). The BabA of specialist strains binds only to glycoconjugates with an unsubstituted terminal Fucα2Gal sequence as in the H type 1 and Le^b determinants, whereas the generalist BabA tolerates a substitution at 3-position of the Gal with an αGal or αGalNAc as in the A or B type 1 and ALe^b or BLe^b determinants.

Here, we further explored the structural requirements for carbohydrate recognition by BabA of generalist and specialist *H. pylori* by isolating and characterizing glycosphingolipids recognized by wild type specialist and/or generalist *H. pylori* but not by the deletion mutant strain lacking the BabA adhesin. The Le^b epitope has only been found in humans, but we initially thought that we had found a porcine Le^b glycosphingolipid when an *H. pylori* BabA-binding glycosphingolipid co-migrating with the Le^b hexaglycosylceramide was detected in the non-acid fraction of blood group O pig intestine. However, after isolation, this BabA-binding glycosphingolipid was characterized as the Globo H hexaglycosylceramide. Further comparative binding studies using our glycosphingolipid collection confirmed that the BabA adhesin in addition to blood group determinants on type 1 core chains recognizes blood group O and A determinants on type 4 core chains with binding to Globo H by both strains and Globo A by the generalist strain. The terminal disaccharides (Fucα2Galβ3) of the H type 1 pentaglycosylceramide and the Globo H hexaglycosylceramide adopt conformations very similar to each other, and this is also the case for the terminal trisaccharides (GalNAcα3(Fucα2)Galβ3) of the A type 1 and the Globo A heptaglycosylceramides (18). These conformational similarities thus explain the binding of BabA to the two sets of isoreceptors.

The enzymatic machinery involved in the biosynthesis of Globo H has not yet been fully elucidated. In humans, there are two functional fucosyltransferases, designated FUT1 and FUT2, that catalyze addition of an α2-linked fucose to a terminal galactose to form the blood group H epitope (for a review, see Ref. 35). These two fucosyltransferases are encoded by two distinct genes, *FUT1* and *FUT2*. FUT1 acts preferentially on type 2 chains, whereas type 1 and type 3 chains and to some extent type 2 chains are acceptors for FUT2. Using siRNAs targeting *FUT1* and *FUT2* in breast cancer stem cells, Chang *et al.* (36) showed that Globo H may be synthesized by both FUT1 and FUT2.

Non-secretor individuals have an increased risk of peptic ulcer disease (37). In these individuals, the precursor of the Le^b sequence, *i.e.* the H type 1 sequence, is not formed due to lack of a functional FUT2 enzyme. Consequently, non-secretors have low amounts of or no Le^b antigens on their epithelial surfaces. However, the Globo H sequence can still be formed by FUT1 and might thus function as an adhesion factor for BabA-expressing *H. pylori* in non-secretor individuals.

The slow migrating BabA-binding fraction P-III was characterized as a mixture of three complex glycosphingolipids (Fucα2Galβ4GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer, Fucα2Galβ3GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer, and Fucα2Galβ4(Fucα3)GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer). The undecaglycosylceramide with an Le^y epitope on the 6-branch and an H type 1 epitope on the 3-branch are to our knowledge novel glycosphingolipid structures. The three compounds in fraction P-III all had an H type 1 determinant on at least one branch, and thus, all three could be recognized by both specialist and generalist BabA.

The binding of the generalist *H. pylori* strain to the nonaglycosylceramide with a repetitive blood group A determinant and an internal type 1 core chain (GalNAcα3(Fucα2)Galβ3-GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer) is a wild card. We have previously found that generalist *H. pylori* strains bind to the ganglio-Le^b hexaglycosylceramide (Fucα2Gal β3(Fucα4)GalNAcβ4Galβ4Glcβ1Cer) (13). Thus, the binding to this nonaglycosylceramide is most likely due to recognition of the terminal A determinant on the ganglio core by the generalist BabA.

Characterization of the binding specificities of the BabA variants is important for understanding the molecular interactions between *H. pylori* and the target host cells. The presence of the BabA-binding Globo H glycosphingolipid in the human stomach was here indicated by the binding of monoclonal antibodies directed against the Globo H determinant to human gastric glycosphingolipids. Thus, Globo H may have a role in the BabA-mediated target tissue adherence of *H. pylori.*

Expression of BabA by *H. pylori* is associated with severe gastric inflammation and an increased risk of developing peptic ulcer or gastric cancer (38, 39). *H. pylori* infects more than half of the world's population, and although the prevalence of infection is decreasing in developed countries, the infection rate is still high in developing countries (40). Furthermore, the treatment options in developing countries are currently inadequate. Targeting BabA might be important for the development of novel treatment strategies against *H. pylori*.

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