

Fut2-null mice display an altered glycosylation profile and impaired BabA-mediated *Helicobacter pylori* adhesion to gastric mucosa

Ana Magalhães², Joana Gomes², Mohd Nazri Ismail³,
Stuart M Haslam³, Nuno Mendes², Hugo Osório²,
Leonor David^{2,4}, Jacques Le Pendu⁵, Rainer Haas⁶,
Anne Dell³, Thomas Borén⁷, and Celso A Reis^{1,2,4}

²Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; ³Division of Molecular Biosciences, Faculty of Natural Sciences, Imperial College London, London, UK; ⁴Medical Faculty, University of Porto, Porto, Portugal; ⁵INSERM, U892, University of Nantes, Nantes, France; ⁶Max von Pettenkofer Institute, LMU München, München, Germany; and ⁷Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, Sweden

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Glycoconjugates expressed on gastric mucosa play a crucial role in host–pathogen interactions. The FUT2 enzyme catalyzes the addition of terminal $\alpha(1,2)$ fucose residues, producing the H type 1 structure expressed on the surface of epithelial cells and in mucosal secretions of secretor individuals. Inactivating mutations in the human *FUT2* gene are associated with reduced susceptibility to *Helicobacter pylori* infection. *H. pylori* infects over half the world's population and causes diverse gastric lesions, from gastritis to gastric cancer. *H. pylori* adhesion constitutes a crucial step in the establishment of a successful infection. The BabA adhesin binds the Le^b and H type 1 structures expressed on gastric mucins, while SabA binds to sialylated carbohydrates mediating the adherence to inflamed gastric mucosa. In this study, we have used an animal model of non-secretors, Fut2-null mice, to characterize the glycosylation profile and evaluate the effect of the observed glycan expression modifications in the process of *H. pylori* adhesion. We have demonstrated expression of terminal difucosylated glycan structures in C57Bl/6 mice gastric mucosa and that Fut2-null mice showed marked alteration in gastric mucosa glycosylation, characterized by diminished expression of $\alpha(1,2)$ fucosylated structures as indicated by lectin and antibody staining and further confirmed by mass spectrometry analysis. This altered glycosylation profile was further confirmed by the absence of Fuc $\alpha(1,2)$ -dependent binding of calicivirus virus-like particles. Finally, using a panel of *H. pylori* strains, with different adhesin expression profiles, we have demonstrated an impairment of BabA-dependent adhesion of *H. pylori* to Fut2-null mice gastric mucosa, whereas SabA-mediated binding was not affected.

Keywords: $\alpha(1,2)$ fucosyltransferase/BabA/bacterial adhesion/*Helicobacter pylori*/Lewis antigens

Introduction

Carbohydrate structures expressed on cell surfaces by glycoproteins, glycolipids, and proteoglycans have been implicated in a wide variety of biological processes such as inflammation, host–pathogen interactions, and tumor metastasis (Varki 1993; Hollingsworth and Swanson 2004). The biosynthesis and modification of protein and lipid glycans are mediated by the concerted action of different glycosyltransferases, including sialyltransferases and fucosyltransferases. In humans, the $\alpha(1,2)$ fucosyltransferase activities are encoded by two closely linked genes corresponding to the *H* blood group locus (*FUT1*) and the *Secretor* locus (*FUT2*). The *FUT2* enzyme catalyzes the addition of terminal $\alpha(1,2)$ fucose residues, producing the H type 1 carbohydrate present on the surface of epithelial cells and in mucosal secretions of secretor individuals (Mollicone et al. 1985). The secretor status depends on the *FUT2* enzyme. Inactivating mutations in the *FUT2* gene, responsible for the nonsecretor phenotype which affect 20% of human population, have been associated with reduced susceptibility to infections by Norwalk virus (Lindesmith et al. 2003), *Campylobacter jejuni* (Ruiz-Palacios et al. 2003), and *Helicobacter pylori* (Ikehara et al. 2001).

H. pylori is a Gram-negative bacterium, specialized in the colonization of human stomach, that infects more than half the world's population (Atherton 2006). The infection is associated with the increased risk to develop gastric lesions, from gastritis to gastric adenocarcinoma (Correa and Houghton 2007). The establishment of a successful infection is dependent on *H. pylori* tight adherence to the mucous epithelial cells and the mucus layer lining the gastric epithelium (Blaser 1993). The *H. pylori* glycan receptors include fucosylated ABO blood group antigens (Boren et al. 1993; Aspholm-Hurtig et al. 2004), glycans with charged groups such as sialic acid (Mahdavi et al. 2002) or sulfate (Guzman-Murillo et al. 2001) and neolacto core chains (Miller-Podraza et al. 2009). The blood group antigen-binding adhesin (BabA) was shown to bind the Lewis b (Fuc $\alpha(2)$ Gal $\beta(3)$ [Fuc $\alpha(4)$]GlcNAc-R) and H type 1 (Fuc $\alpha(2)$ Gal $\beta(3)$ GlcNAc-R) histo-blood group carbohydrate structures expressed in gastric epithelium and mucins (Ilver et al. 1998). Later, a sialic-acid binding adhesin (SabA) that recognizes sialylated carbohydrates was identified (Mahdavi et al. 2002). SabA mediates the adherence of *H. pylori* to inflamed gastric mucosa by binding carbohydrate structures such as Sialyl-Lewis x (NeuAc $\alpha(3)$ Gal $\beta(4)$ [Fuc $\alpha(3)$]GlcNAc-R) and Sialyl-Lewis a (NeuAc $\alpha(3)$ Gal $\beta(3)$ [Fuc $\alpha(4)$]GlcNAc-R) (Mahdavi et al. 2002; Aspholm et al. 2006).

Mucin-type *O*-glycans, including those described above, play a crucial role in the pathogenesis process of *H. pylori* infection (Kobayashi et al. 2009). Therefore, it is expected that the diversity of carbohydrate expression on gastric mucosa observed

¹To whom correspondence should be addressed: Tel: +351-225570700; Fax: +351-225570799; e-mail: celsor@ipatimup.pt

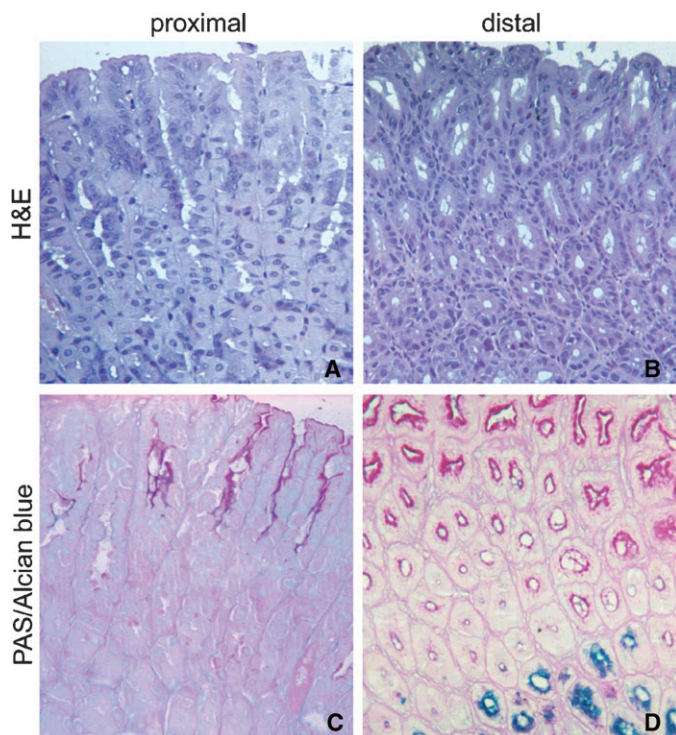


Fig. 1. Histological characterization of wild-type and Fut2-null mice gastric mucosa. Paraffin-embedded sections from 6- to 8-week-old wild-type and Fut2-null mice stomachs were used for the hematoxylin/eosin (H&E) (A and B) and the periodic acid schiff (PAS)/alcian blue staining (C and D). Since no major differences were observed between wild-type and Fut2-null mice, only wild-type mice gastric mucosa staining is represented in the figure. Magnification 200 \times .

among individuals may confer distinct susceptibilities to *H. pylori* colonization and infection. In humans, this diversity can be attributed to several genetic polymorphisms in glycosyltransferase genes together with host immune response to pathogens. For this reason, genetically modified mice are an important tool to study the role of a specific glycosyltransferase in the biosynthesis of glycans involved in bacterial adhesion.

Domino et al. have developed a homozygous Fut2-null mouse model, by replacing Fut2 with the bacterial reporter gene *lacZ* (Domino, Zhang, Gillespie, et al. 2001). The mouse Fut2 gene is the ortholog of the human *secretor* gene and it has been shown that the FUT2 enzyme is responsible for $\alpha(1,2)$ fucosylation in the mouse gastrointestinal tract (Domino, Zhang, Lowe 2001; Hurd et al. 2005).

In this study, we have evaluated alterations in the glycosylation profile of Fut2-null mice gastric mucosa in comparison with the wild-type mice, using lectins, a panel of monoclonal antibodies for Lewis antigens, and mass spectrometry analysis. We further analyzed whether these modifications would have a role in *H. pylori* adhesion and observed that *H. pylori* strains expressing a functional BabA adhesin showed decreased adhesion to Fut2-null mice gastric mucosa. This observation was further supported by the fact that this decrease was not observed when testing strains that only express the sialic-acid binding adhesin SabA, showing that this impaired adhesion could be strictly attributed to reduced expression of BabA ligands in Fut2-null mice gastric mucosa.

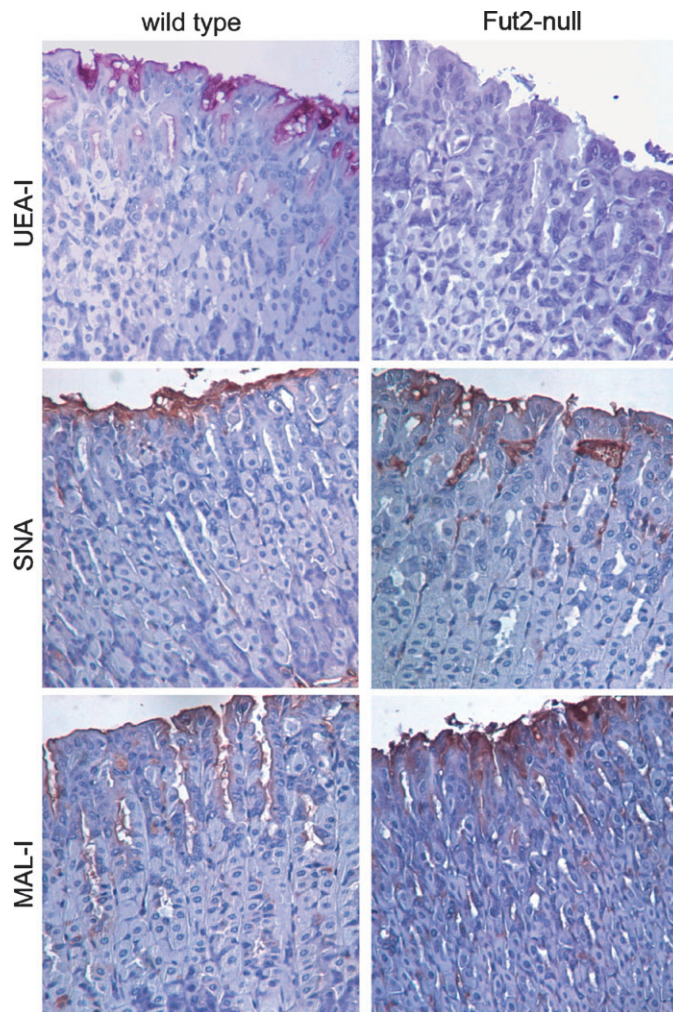


Fig. 2. Lectin staining of wild-type and Fut2-null mice gastric mucosa. Paraffin-embedded sections from wild-type and Fut2-null mice gastric mucosa were used to evaluate the staining of the biotinylated lectins: UEA-I (*Ulex europaeus*), SNA (*Sambucus nigra*), and MAL-I (*Maackia amurensis* lectin I). The specificity of the lectins is described in Table I. Magnification 200 \times .

Results

Histology and lectin staining of wild-type and Fut2-null mice gastric mucosa

No histological differences were observed when comparing the hematoxylin and eosin-stained gastric mucosa of wild-type mice with the Fut2-null mice. The PAS staining reveals the presence of neutral mucins in the superficial mucous cells of the foveolar epithelium and in most cells of pyloric glands both in wild-type and Fut2-null mice (Figure 1). The superficial mucous cells of the foveolar epithelium and most cells from the antrum (distal) pyloric glands also stained positive for sialomucins, as indicated by the alcian blue staining (Figure 1). Sialomucins were also detected in glands of the proximal stomach.

The major difference detected was a complete loss of binding of the $\alpha(1-2)$ fucose-specific lectin UEA-I to Fut2-null mice (Figure 2, Table I). No major alterations in gastric mucosa sialylation were observed. The binding of the $\alpha 2-6$ -specific SNA lectin was similar in both types of mice (Figure 2, Table I). Staining with MAL-I lectin, which recognizes the products of $\alpha 2,3$

Table I. Specificity of lectins used for immunohistochemistry

Lectin	Dilution	Binding specificity	References
UEA-I (<i>Ulex europaeus</i>)	(1:50)	Fuc α 1,2(Gal β 1,4GlcNAc)	(Mollicone et al. 1985)
SNA (<i>Sambucus nigra</i>)	(1:250)	Sia α 2,6(Gal or GalNAc)	(Shibuya et al. 1987; Mattox et al. 1992)
MAL-I (<i>Maackia amurensis</i> lectin I)	(1:250)	Sia α 2,3(Gal β 1,4GlcNAc) or Gal β 1,4GlcNAc	(Wang and Cummings 1988; Knibbs et al. 1991)

sialyltransferases, showed a minor increase in Fut2-null mice when comparing with the wild-type gastric mucosa (Figure 2, Table I).

Fut2-null mice gastric mucosa immunoreactivity with Lewis monoclonal antibodies

Using a panel of different monoclonal antibodies (mAbs) specific for Lewis antigens (Table II), it was possible to observe different patterns of staining between wild-type and Fut2-null mice. Le^a antibodies, Ca3F4 and 7LE, strongly stained the surface mucous cells of Fut2-null mice but only weakly stained a few cells in wild-type mice (Figure 3A and B). In contrast, BG6 and 2.25LE, Le^b recognizing antibodies, bound to superficial mucous cells of the foveolar epithelium of wild-type mice but not to the gastric mucosa of Fut2-null mice (Figure 3C–F). The pattern of staining of the 2.25LE mAb was restricted to some cells and less intense when compared with BG6 staining. Binding of the Le^a (Ca3F4) and Le^b (BG6) mAbs to gastric mucosa was specifically blocked by preincubation of the mAb with the corresponding soluble purified Le^a or Le^b oligosaccharide (supplementary Figure S1). No staining was observed with the CA19.9, anti sialyl-Le^a, antibody (Figure 3G and H).

Wild-type and Fut2-null mice gastric mucosa were stained in a similar manner with SH1 mAb. Le^x expression was observed both on the surface mucous cells and in most of the neck cells and pyloric glands (Figure 3I and J). The intensity and extent of staining of AH6 mAb, anti Le^y, was higher in wild-type than in Fut2-null mice. In null mice gastric mucosa, the staining of AH6 was restricted to mucous cells of the foveolar epithelium (Figure 3K and L). No major differences were observed between wild-type and Fut2-null mice regarding the staining of KM93, specific for sialyl-Le^x, and FH6, that recognizes (dimeric)sialyl-Le^x. Both antibodies showed reactivity with the surface mucous

cells, as well as few cells from the antrum glands of both wild-type and Fut2-null mice (Figure 3M–P).

Mass spectrometry analysis of mice gastric mucosal scrapings

Murine gastric mucosal scrapings were prepared for glycomic screening according to the methodology described in *Material and methods*. In summary, *O*-glycans were released from tryptic digests of mucosal homogenates and were permethylated prior to mass spectrometric analysis. *O*-Glycomic profiles of samples from both wild-type and Fut2-null mice were acquired by MALDI-TOF, and components of interest were sequenced by collision-induced tandem mass spectrometry using both MALDI and Electrospray ionization methods.

Representative MS and MS/MS data from these experiments are shown in Figure 4. Glycan structures were assigned based on their mass value, the fragment ion data, and knowledge of the biosynthetic pathways of mucin-type *O*-glycosylation.

The gastric mucosal scraping from wild-type mice shows *O*-glycans with compositions up to 12 monosaccharides (Figure 4A). The most abundant types of *O*-glycan are core 2 structures, many of which are branched on their core 1 arm, as noted in an earlier study of murine stomach glycans (Stone et al. 2009). The terminal epitopes are dominated by blood group H type 2-antigens (Fuc α 2Gal β 4GlcNAc), for instance *m/z* 2404. However, some of the larger glycans, for example *m/z* 2578, carry difucosylated antenna. The MS/MS data for this component gave evidence for Le^y, by showing that one antenna is difucosylated and that there was a convincing signal for the elimination of fucose from the molecular ion, confirming that a portion of the fucose in the glycan is attached to position 3 of GlcNAc (data not shown).

As expected, the MALDI profiles of gastric mucosal scrapings from Fut2-null mice, when compared with wild type, showed a major loss of glycans carrying the H-epitope

Table II. Specificity of monoclonal antibodies used for immunohistochemistry

Ab clone	Isotype	Dilution	Antigen (reference)	Described cross-reactivity (reference)
Ca3F4	IgG2a	1:5	Le ^a (Young et al. 1983)	–
7LE	IgG1	1:10	Le ^a (Daher et al. 1987)	–
BG6 (Signet T218)	IgM	1:50	Le ^b (Sakamoto et al. 1986)	Le ^c (Monteiro et al. 1998) H type 1 (Monteiro et al. 1998; Manimala et al. 2007) Le ^y (Manimala et al. 2007)
2.25LE	IgG1	1:10 000	Le ^b (Bara et al. 1986)	Le ^a (Good et al. 1992; Amano et al. 1997; Manimala et al. 2007)
CA19-9 (Santa Cruz)	IgG1	1:500	sLe ^a (Pour et al. 1988)	–
SH1	IgG3	1:5	Le ^x (Fukushi, Hakomori, et al. 1984)	–
AH6	IgM	1:10	Le ^y (Abe et al. 1983)	–
FH6	IgM	1:10	sLe ^x (Fukushi, Nudelma, et al. 1984)	–
KM93 (Calbiochem)	IgM	1:60	sLe ^x (Sasaki et al. 1993)	–

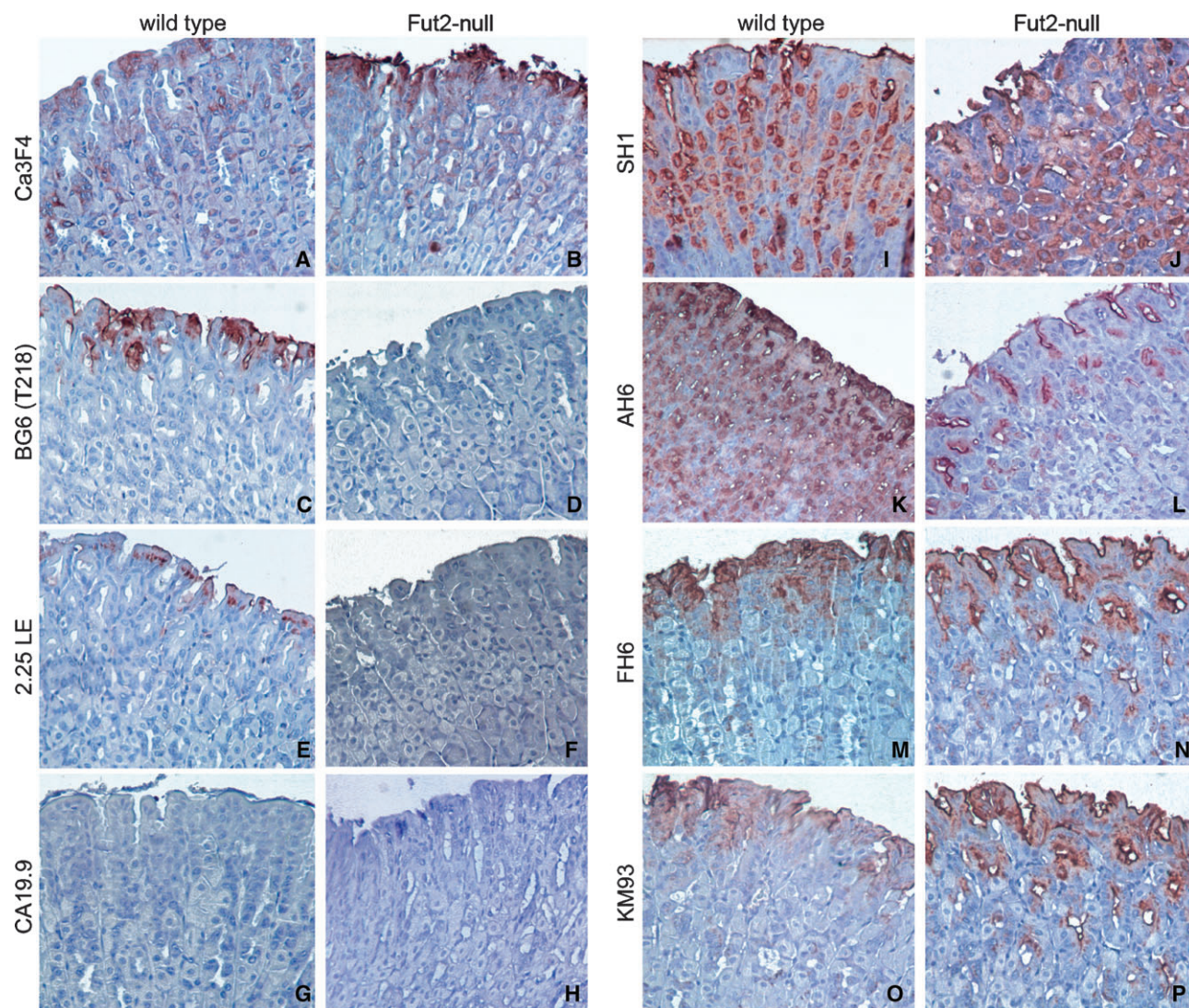
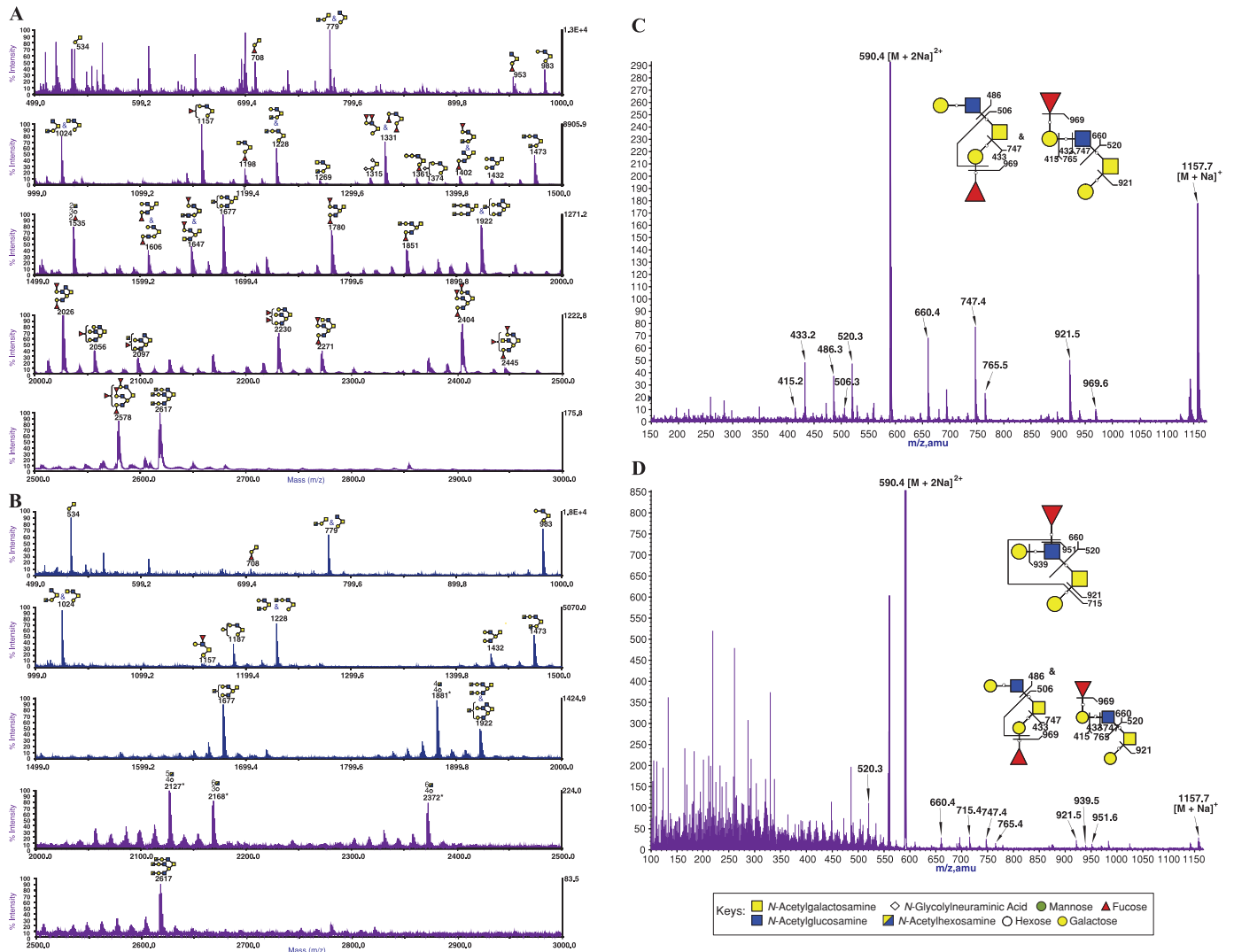


Fig. 3. Immunohistochemical staining of wild-type and Fut2-null mice gastric mucosa using a panel of anti-Lewis mAbs. Paraffin-embedded sections from wild-type and Fut2-null mice gastric mucosa were stained using a panel of Lewis recognizing antibodies. Specificity of the monoclonal antibodies used for immunohistochemistry as well as previously shown cross reactivity are described in Table II. Magnification 200 \times .

(Figure 4B). However, it is possible to observe minor fucosylated terminal galactose, as demonstrated by m/z 708 (Figure 4B), which on MS/MS analysis gave the same fragment ions (the major ions are m/z 298, 433 and 520, data not shown) as the H-core 1 sequence found in the wild type, indicating that a residual $\alpha(1,2)$ fucosyltransferase activity remains in the stomach of the null mice.

Both wild-type and Fut2-null samples gave a MALDI molecular ion at m/z 1157 whose composition is compatible with putative Lewis structures. In order to establish whether a Lewis structure contributes to this molecular ion, MS/MS experiments were carried out on the corresponding doubly charged ion at m/z 590.4 in the electrospray spectra. These experiments revealed that the dominant wild-type glycans carry the H-epitope on either the core 1 or the core 2 arm (Figure 4C). In the latter case, the fragment ions are consistent with the H-antigen being of the type 2 sequence in which the Gal is attached to the 4-position of GlcNAc. However, we cannot rule out minor

amounts of the type 1 sequence. Also, because of the dominant H-containing structures it is not possible to determine whether any Lewis structure is contributing to m/z 1157 in the wild type. In contrast, the Fut2-null mice showed firm evidence for the Le^x structure, despite the relevant glycan (m/z 1157 in the MALDI data) being only a small fraction of a percent of the total glycan mixture (Figure 4B). Because of the very low abundance of this component, contaminant signals are prominent below m/z 500 in the electrospray MS/MS spectrum (Figure 4D). Fortunately, however, the region of the MS/MS spectrum which contains structurally useful fragment ions (above m/z 600) is relatively free from contaminating ions. Especially relevant are the signals at m/z 951 and m/z 715 (Figure 4D) because they correspond to elimination of fucose and separate eliminations of fucose plus hexose, respectively. As shown on the cartoon annotations, these signals can only be derived from a Le^x structure (Figure 4D). Nevertheless, some of the H-structure is also present because m/z 747 can only be derived from a glycan which carries the



fucose attached to galactose because this ion is produced by elimination of fucose plus hexose via a single cleavage event (see cartoon annotations).

Fut2-null mice gastric mucosa shows no binding of Calicivirus virus-like particles

Since calicivirus virus-like particles (VLPs) have been shown to bind gastroduodenal epithelial cells from secretor individuals but not to cells from nonsecretors, deficient in the *FUT2* gene, we further evaluated VLPs binding capacity to *Fut2*-null mice gastric mucosa (Marionneau et al. 2002; Lindesmith et al. 2003).

As shown in Figure 5, DVLP (Dijon strain VLP) and NVLP (Norwalk strain VLP) bound the surface mucous cells of wild-type gastric mucosa but no binding was observed to the gastric mucosa of *Fut2*-null mice. Negative controls were performed by replacing the incubation with VLPs by PBS and no nonspecific binding of the VLPs recognizing antibodies could be detected.

BabA-mediated *H. pylori* adhesion is impaired in *Fut2*-null mice gastric mucosa

H. pylori strains 17875/Leb, 17875*babA1A2* mutant, and J99 have been previously characterized regarding *BabA* and *SabA* expression (Ilver et al. 1998; Mahdavi et al. 2002; Aspholm et al. 2006). As shown in Figure 6A, 17875/Leb and J99 strains express both *BabA* and *SabA* adhesins while the 17875*babA1A2* mutant strain expresses *SabA* adhesin only. It has been demonstrated that besides expressing both *BabA* and *SabA* adhesins, the 17875/Leb strain is unable to bind to sialylated antigens (Mahdavi et al. 2002).

To better understand the importance of *FUT2* enzymatic activity in the biosynthesis of potential *H. pylori* ligands, we evaluated the binding capacity of these strains to the gastric mucosa of *Fut2*-null mice in comparison with wild-type mice. As shown in Figure 6B and C, 17875/Leb strain bound the surface mucous cells of wild-type mice but no adhesion was observed to the gastric mucosa of *Fut2*-null mice. The *SabA* competent

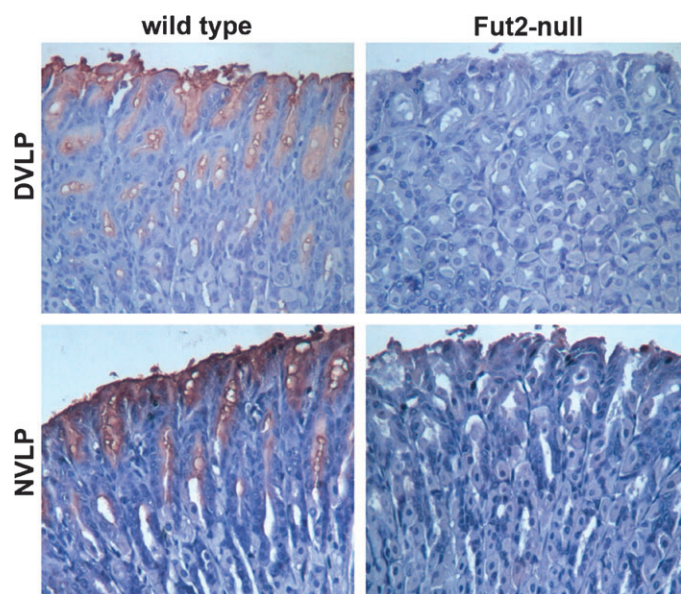


Fig. 5. Calicivirus virus-like particles binding to wild-type and Fut2-null mice gastric mucosa. Evaluation of DVLP (Dijon virus-like particle) and NVLP (Norwalk virus-like particle) binding to paraffin-embedded gastric sections from wild-type and Fut2-null mice. Magnification 200 \times .

17875babA1A2 strain adhered equally well to the surface mucous cells of wild-type and Fut2-null mice and, in addition, quantification revealed that an increased number of bacteria bound the glandular region of Fut2-null mice gastric mucosa when compared to the wild type. The J99 strain, that expresses both BabA and SabA adhesins (Figure 6A), showed decreased adhesion to superficial mucous cells of the foveolar region of Fut2-null mice when compared with the wild type.

Besides these largely characterized model *H. pylori* strains, we further evaluated the adhesion of a panel of strains from clinical isolates. These strains were characterized for BabA and SabA expression as shown in Figure 7A. Quantification of adhered bacteria showed that adhesion of the BabA-positive strains, 094UK 127UK and BO265, was decreased in the Fut2-null mice gastric mucosa when compared to the wild-type mice (Figure 7B). *H. pylori* 101UK strain was an exception because although being considered BabA positive, binding was not affected by the Fut2 deficiency (Figure 7B). The exclusively SabA-positive strains, 111UK and 123UK, adhered equally to the gastric mucosa of both wild-type and Fut2-null mice. As expected, the levels of adhesion of strains 097UK and 131UK that did not express any of the adhesins were very low.

Discussion

Glycoconjugates expressed on the gastric mucosa play a crucial role in the establishment of a close contact between *H. pylori* and mucous epithelial cells favoring bacterial adhesion and colonization of gastric mucosa (Ilver et al. 1998; Mahdavi et al. 2002; Kobayashi et al. 2009). In humans, the FUT2 enzyme is responsible for the biosynthesis of the lacto-series type 1 (A1, B1, H1, Le^b) and type 2 (A2, B2, H2, Le^y) in the gastric superficial epithelium (Mollicone et al. 1985). It has been demonstrated by our group and others that infection by *H. py-*

lori is influenced by the secretor phenotype, which is defined by the presence of an active FUT2 (secretor) enzyme (Ikehara et al. 2001; Lee et al. 2006; Azevedo et al. 2008). In this study, we have used an animal model of nonsecretors, Fut2-null mice, to characterize the glycosylation profile of the wild-type and null mice gastric mucosa and to evaluate the effect of the observed glycan expression modifications in the process of *H. pylori* adhesion to gastric epithelial cells.

First we characterized the alterations in the glycosylation profile of Fut2-null mice gastric mucosa compared to wild-type mice. The surface epithelium of wild-type gastric mucosa stained positive with the α (1-2)fucose-specific lectin UEA-I, whereas complete loss of staining in the Fut2-null mice was observed (Figure 2). This result is in accordance with the absence of UEA-I staining in the uterine epithelia of Fut2-null mice, described by Domino, Zhang, Gillespie, et al. (2001).

Unexpectedly, we observed that both BG6 (T218) and 2.25LE, Le^b recognizing antibodies, bound to the superficial mucous cells of wild-type gastric mucosa but not to Fut2-null mice (Figure 3C–F). This staining was not expected since mice lack an ortholog of the human *FUT3* gene and no α 1,4fucosyltransferase enzyme has ever been described in mice (Falk et al. 1995; Nairn et al. 2008). The mouse gene orthologous to the ancestral gene for human FUT3, FUT5, and FUT6 seems to be a pseudogene (Gersten et al. 1995; Smith et al. 1996; Costache et al. 1997).

Moreover Falk et al. (1995) have showed de novo expression of Le^b, detected with a monoclonal antibody 96 FR2.10, by expressing a human α 1,3/4fucosyltransferase enzyme in the gastric pit cell lineage of FVB/N transgenic mice (Falk et al. 1995). Recently, characterization of glycosphingolipid structures from these FVB/N transgenic mice identified a Le^b-like glycosphingolipid (Fuc α 2Gal β 3[Fuc α 4]GalNAc β 4Gal β 4Glc β 1Cer) that was recognized by 96 FR2.10 but not by a BG6 (T218) antibody (Fagerberg et al. 2009). The same study indicated that the Le^b epitope present in glycoproteins from the transgenic Le^b mouse stomach can differ from the “traditional” Le^b epitope because it is not recognized by the BG6 (T218) antibody (Fagerberg et al. 2009). One possible interpretation for the staining of wild-type mice gastric mucosa we observed with the BG6 (T218) and 2.25LE, Le^b recognizing antibodies, is antibody cross-reactivity since it has been previously demonstrated that BG6 (T218) cross-reacted significantly with Le^c, Le^y, and blood group H1 (Monteiro et al. 1998; Manimala et al. 2007), whereas the 2.25LE antibody displayed weak cross-reactivity with Le^a (Good et al. 1992; Amano et al. 1997; Manimala et al. 2007), although for the latter that would not explain the loss of binding to Fut2-null mice tissue sections. Noteworthy and also unexpected was the increased binding of the Le^a antibodies Ca3F4 and 7LE to the surface mucous cells of Fut2-null mice, suggesting that lack of α (1,2)fucosyltransferase activity would lead to accumulation of Le^a structures (Figure 3A and B). Once again, possible antibody cross-reactivity may be considered, even though the 7LE antibody displayed excellent specificity within the context of the carbohydrate microarray performed by Manimala et al. (2007). Taken together, anti-Le^a and anti-Le^b antibody binding patterns suggest expression of Lewis type 1 structures in mice gastric mucosa corroborated by antibody inhibition using specific synthetic saccharides. However, mass spectrometry analysis of the *O*-glycans of mucosal scrapings could not detect these structures indicating that, if present, the levels are

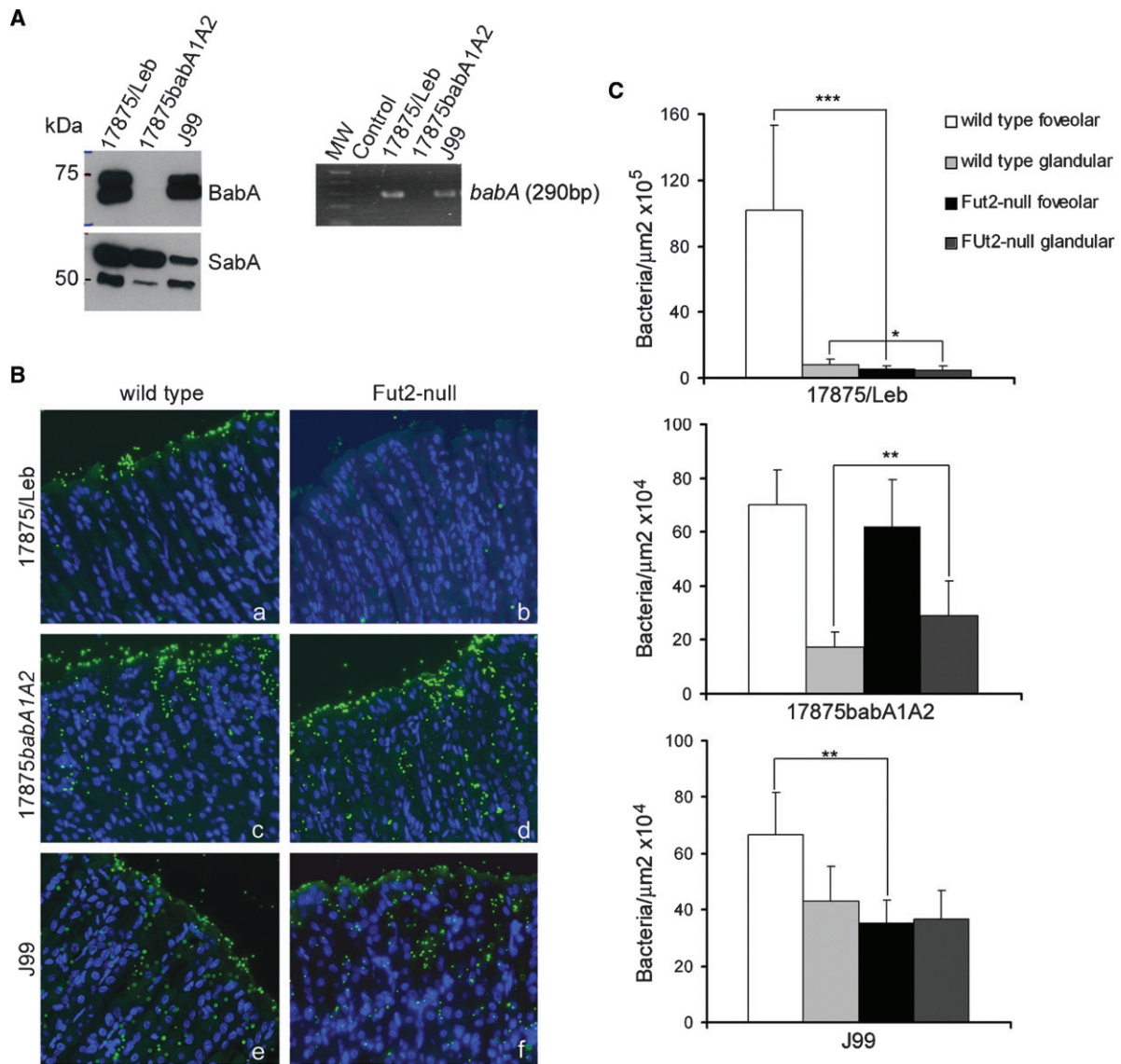


Fig. 6. Evaluation of adhesion of the *H. pylori* model strains 17875/Leb, 17875babA1A2, and J99 to the gastric mucosa of wild-type and Fut2-null mice. (A) Immunoblot analysis of *H. pylori* strains using BabA and SabA antibodies and evaluation of *babA* gene expression by PCR. (B) Adhesion of fluorescein-labeled *H. pylori* strains 17875/Leb, 17875babA1A2 and J99 to the gastric mucosa of wild-type (a, c, e) and FUT2-null mice (b, d, f). Magnification 200 \times . (C) Quantification of adhesion of *H. pylori* strains 17875/Leb, 17875babA1A2, and J99 to the gastric mucosa of wild-type and Fut2-null mice. Each value represents the mean of three different fields within the foveolar or the glandular regions, as defined in *Material and methods*, of two wild-type or Fut2-null mice stomachs; significance was evaluated using the Student's *t*-test. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

extremely low. Further structural studies will be performed using gastric mucosa samples enriched by using anti-Le^a and anti-Le^b binding proteins.

Regarding type 2 structures, the pattern of Le^x expression detected with the monoclonal antibody SH1 was similar in both wild-type and null mice (Figure 3I and J). However, Le^x could only be detected in Fut2-null mice, for example *m/z* 1157 (Figure 4D). This could be explained by the fact that, contrary to the wild type, this sample is not dominated by H-epitopes. Therefore, it is possible to turn up the gain for detecting glycans bearing Le^x which are originally low in abundance. Decreased Le^y expression was detected in Fut2-null mice, both by immunohistochemistry using an AH6 monoclonal antibody (Figure 3K and L) and mass spectrometry analysis (Figure 4A), suggesting that the FUT2 enzyme

is participating in the fucosylation of both type 1 and type 2 structures.

We observed that both wild-type and Fut2-null mice stained positively with KM93, directed to sialyl-Le^x, and FH6 that recognizes (dimeric)sialyl-Le^x. Since the FH6 monoclonal antibody has been shown to recognize the *N*-acetylneuraminyl Le^x oligosaccharide but not its *N*-glycolyl form (Mitoma et al. 2009), our results suggest that *N*-acetylneuraminyl Le^x is being detected. The presence of sialyl-Le^x was also described in the gastric mucosa of FVB/N Le^b transgenic mice (Mahdavi et al. 2002).

The MALDI profile of wild-type mice gastric mucosal scrapings identified the Fuc-Gal-epitope (blood group H-type epitope) as the most abundant monofucosylated glycan. This observation is in accordance with the recently described analysis of

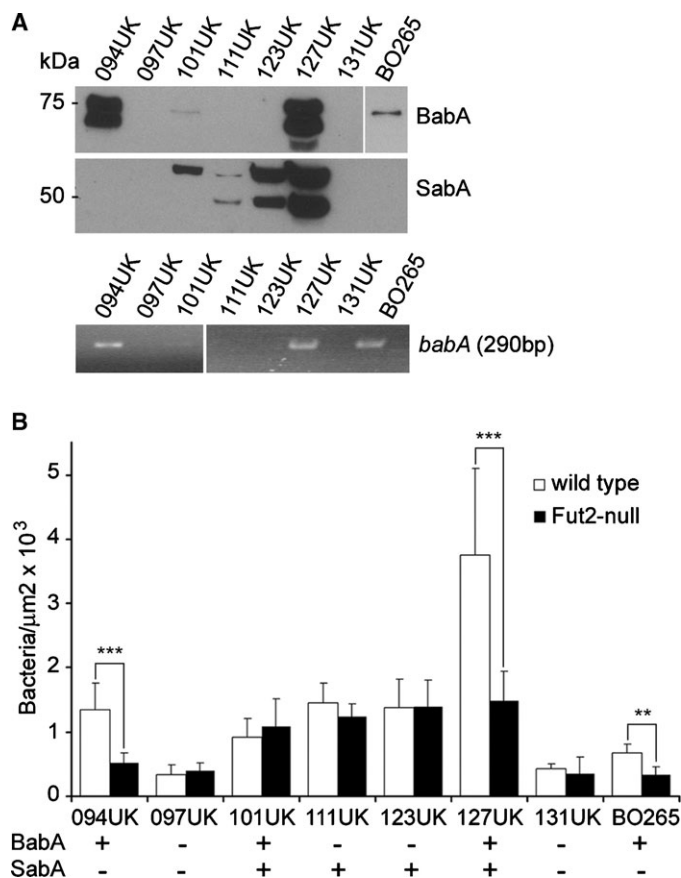


Fig. 7. Evaluation of adhesion of a panel of *H. pylori* strains with different adhesins expression profile to the gastric mucosa of wild-type and Fut2-null mice. (A) Immunoblot analysis of *H. pylori* strains 094UK, 097UK, 101UK, 111UK, 123UK, 127UK, 131UK, and BO265 using BabA and SabA antibodies and evaluation of *babA* gene expression by PCR. (B) Quantification of adhesion of *H. pylori* strains 094UK, 097UK, 101UK, 111UK, 123UK, 127UK, 131UK, and BO265 to the gastric mucosa of wild-type and Fut2-null mice. Each value represents the mean of five different fields of two wild-type or Fut2-null mice stomach; significance was evaluated using the Student's *t*-test. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

O-linked oligosaccharides present in mice cervical mucins that also identified this structure as being highly expressed (Domino et al. 2009). As expected, Fut2-null mice showed major loss of glycans carrying the H-epitope. However, minor terminal fucosylation could be detected in Fut2-null mice gastric mucosa as demonstrated by the molecular ions m/z 708 and fragment ion m/z 747 (Figure 4B and D, respectively) and the binding of the AH6 antibody. This $\alpha(1,2)$ fucosylation activity may be attributed to mouse Fut1 since fucosyltransferase activity assays using extracts from COS-7 cells expressing mouse Fut1 demonstrated that this murine enzyme can use both type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) soluble acceptors in vitro (Domino et al. 1997). Northern-blot analysis showed expression of Fut1 transcript in adult mouse stomach tissue, although an in situ hybridization technique did not reveal specific hybridization in stomach cells (Domino et al. 1997).

The different glycan profile of Fut2-null mice gastric mucosa could be further confirmed by the lack of binding of calicivirus VLPs of the Norwalk and Dijon strains. VLPs have been shown to bind H-type 1 structures present on gastroduodenal epithelial

cells from secretor individuals but not on cells from nonsecretors (Marionneau et al. 2002; Lindesmith et al. 2003), indicating that Fut2-null mice surface mucous cells mimic nonsecretor gastric epithelial cells.

Binding of *H. pylori* to the fucosylated blood group antigens Le^b and H-type 1 is mediated by the blood group antigen-binding adhesin BabA, an outer membrane protein expressed by most disease causing *H. pylori* strains (Ilver et al. 1998; Gerhard et al. 1999). After colonization, *H. pylori* is able to upregulate a set of inflammation-associated genes, including the β 3GnT5 enzyme that participates in the synthesis of sLe^x (Marcos et al. 2008). SabA adhesin binds sLe^x and also other structurally related sialylated antigens contributing to the persistence of *H. pylori* infection (Mahdavi et al. 2002).

H. pylori reference strain J99 expresses both BabA and SabA active adhesins with binding affinity for Le^b, H1, blood group A-Le^b, blood group A-H1, sialyl-Le^x, sialyl-di-Le^x, sialyl-Le^a, 3'-sialyl-lactose, and sialyl-lactosamine (Aspholm-Hurtig et al. 2004; Aspholm et al. 2006). We observed that FITC labeled J99 strain bound to surface mucous cells and glands of both wild-type and Fut2-null mice; however, levels of adhesion to the surface mucous cells were decreased in Fut2-null mice. In order to evaluate if this decrease in adhesion could be attributed to decreased expression of $\alpha(1,2)$ fucosylated glycans recognized by BabA adhesin, we evaluated the adhesion of the 17875/Leb strain, which besides expressing both BabA and SabA adhesins recognizes Le^b, H1, blood group A-Le^b, blood group A-H1, and blood group B-Le^b but does not bind to sialylated glycans (Aspholm-Hurtig et al. 2004; Aspholm et al. 2006). Binding of the 17875/Leb strain was restricted to the surface mucous cells of foveolar epithelium of wild-type mice and completely absent in the null mice. In addition, we have tested a BabA mutant strain 17875babA1A2, with affinity for sialyl-Le^x, sialyl-di-Le^x, sialyl-Le^a, 3'-sialyl-lactose, and sialyl-lactosamine (Aspholm-Hurtig et al. 2004; Aspholm et al. 2006) and observed that this strain bound similarly to wild-type and Fut2-null mice foveolar and surface mucous cells. Interestingly, this strain adhered more to the glandular region of Fut2-null mice in comparison with the wild type. This observation could be explained by the fact that these mice show decreased H-type 2 expression, as further demonstrated by mass spectrometry, so it is possible that they accumulate increased amounts of sialylated-type 2 structures. Similarly, it has been previously reported a reciprocal induction in fucosylation versus sialylation during infection by *H. pylori* of secretor positive macaques and that this reciprocity was much lower in weak secretor individuals (Lindén et al. 2008).

We further evaluated the adhesion of a larger panel of *H. pylori* strains and we observed that adhesion of BabA-positive strains, 127UK, BO265, and 094UK, was significantly decreased in the Fut2-null mice gastric mucosa when compared to the wild-type mice (Figure 7B). *H. pylori* 101UK strain did not seem to be affected by the FUT2 deficiency, suggesting that adhesion of this strain is mostly mediated by SabA, although by Western blot it was possible to detect a faint band corresponding to BabA adhesin (Figure 7A and B). No significant differences were observed for SabA-positive strains, 111UK and 123UK. *H. pylori* strains 097UK and 131UK that did not express any of the adhesins did not adhere to either wild-type or mutant mice gastric mucosa.

In summary, we have demonstrated that Fut2-null mice show marked alteration in gastric mucosa glycosylation patterns,

revealed by the absence of UEA-I lectin binding, increased Le^a immunostaining, decreased Le^b antibodies immunoreactivity, and diminished H-type 2 and Le^y expression as detected by mass spectrometry. Furthermore, we have shown that this altered glycosylation profile of the Fut2-null mice gastric mucosa completely abolishes the binding of calicivirus VLPs. Finally, using a panel of *H. pylori* strains, we have demonstrated that whereas SabA-mediated binding was not affected, BabA-dependent adhesion was impaired in the Fut2-null mice. Our results confirm the relevance of BabA adhesin and the alpha2-linked fucose residues in *H. pylori* binding to host gastric mucosa and set the ground for the use of this model in studies addressing *H. pylori* adhesion.

Material and methods

Mice models

Wild-type C57BL/6 and FUT2-null mice (Domino, Zhang, Gillespie, et al. 2001) were obtained from the Consortium for Functional Glycomics (CFG). Mice were reproduced, maintained, and housed at IPATIMUP Animal House at the Medical Faculty of the University of Porto and handled accordingly to the Guidelines for the Care and Use of Laboratory Animals, directive 86/609/EEC. Mice genotyping was performed as previously described, using primers specific for the wild-type and the null alleles (supplementary Figure S2) (Domino, Zhang, Gillespie, et al. 2001).

Tissue samples and histology

Stomachs from 6- to 8-week-old wild-type and null mice were harvested and processed for paraffin sections. Every specimen was fixed in 10% formalin and embedded in paraffin wax. Serial sections were cut and used for histochemistry and immunohistochemistry. Tissue sections from all mice were stained with hematoxylin and eosin (H&E) for morphological analysis. Alcian blue (pH 2.5)/periodic acid-Schiff technique was used to identify neutral and sialomucins.

Lectin staining

Paraffin sections, after deparaffination, rehydration, and blocking of endogenous peroxidase activity with 3% H₂O₂ in methanol, were incubated with 10% of BSA in PBS for 30 min at room temperature. Sections were then incubated with one of the biotinylated lectins UEA-I (*Ulex europaeus*, SIGMA), SNA (*Sambucus nigra*, Vector Laboratories), or MAL-I (*Maackia amurensis* lectin I, Vector Laboratories) for 1 h. The slides were subsequently washed in PBS and incubated for 30 min with avidin-biotin complex (Vectastain Elite ABC kit, Burlingame, CA) according to manufacturer's recommendations. Staining was performed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) containing 0.02% hydrogen peroxide. Slides were counter-stained with Mayer's hematoxylin. Negative controls were performed by replacing lectin with PBS.

Immunohistochemistry

Monoclonal antibodies used in this study, their specificity, and their references are listed in Table II. Immunohistochemical staining was performed similarly to the lectin staining with the following alterations: sections were blocked with normal rabbit

serum diluted 1:5 in PBS containing 10% BSA, and then incubation with the monoclonal antibody was performed overnight at 4°C; next, slides were washed in PBS and incubated for 30 min with a biotinylated rabbit anti-mouse antibody (E0354-DakoCytomation, Glostrup, Denmark) diluted 1:200 in PBS containing 5% of BSA prior to incubation with HRP-avidin-biotin complex. Negative controls were performed by replacing primary antibody with PBS.

Sample preparation for mass spectrometric analysis

Scrapings from gastric mucosa of 6 to 8-week-old mice were prepared for glycomic analysis according to methodology that have been modified and optimized from a previously reported methodology (Sutton-Smith et al. 2000; Jang-Lee et al. 2006). In brief, mice gastric mucosa scrapings were homogenized with ice-cold water. This is followed by extraction of proteins and glycoproteins which were then sequentially reduced carboxymethylated and digested with trypsin (Sigma-Aldrich, UK). O-Glycans were released from glycopeptides by reductive elimination using KBH₄ in KOH. After purification on Dowex columns (Sigma-Aldrich), the O-glycan samples were permethylated and then further purified with Sep-Pak cartridges. O-Glycans were eluted in aqueous acetonitrile fractions and then lyophilized. Glycans are normally eluted in the 35% and 50% acetonitrile fractions; therefore, only these fractions were subjected to mass spectrometric analysis.

Mass spectrometric data acquisition

Permethylated samples were dissolved in 10 µL of methanol and 1 µL of the dissolved sample was pre-mixed with 1 µL of matrix (20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 70% (v/v) aqueous methanol) (Sigma-Aldrich) spotted onto a target plate. MALDI mass spectrometric (MS) and tandem mass spectrometric (MS/MS) data were acquired on a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, UK) in the reflectron mode. The potential difference between the source acceleration voltage and the collision cell was set to 1 kV and argon was used as collision gas. The 4700 Calibration Standard kit (Applied Biosystems, UK) was used as the external calibrant for the MS mode and [Glu1]fibrinopeptide B human was used as an external calibrant for the MS/MS mode. Tandem mass spectrometric data were also acquired by using a QSTAR Pulsar-I mass spectrometer (Applied Biosystems, UK). Permethylated samples were dissolved in 10 µL of methanol and 1 µL of the dissolved sample was introduced into the electrospray source of the mass spectrometer via a nanoflow electrospray needle. Nitrogen was used as the collision gas with the pressure being maintained at 5.3×10^{-5} Torr and collision energies typically varied from 35 to 90 eV.

Virus-like particle (VLP) binding to mice gastric mucosa

VLPs from the norovirus strains Norwalk (NVLPs) and Dijon 171/96 (DVLPs) of the GI.1 and GII.4 Grimsby type, respectively, as well as their respective antibodies were prepared as previously described (Rydell et al. 2009). Paraffin sections, after deparaffination, rehydration, and blocking of endogenous peroxidase activity as described above, were incubated with 3% BSA in PBS for 30 min. Then sequential sections were incubated with either NVLP (Norwalk VLP), 5 µg/mL in PBS for 90 min at room temperature, or DVLP (Dijon VLP) 3 µg/mL in

PBS containing 1% BSA overnight at 4°C. After three washes with PBS, sections were incubated with a rabbit polyclonal antibody specific for each virus particle for 1 h at room temperature. Next, samples were incubated with goat anti rabbit-HRP (Santa Cruz Biotechnology, CA) and detection was achieved with 3,3'-diaminobenzidine tetrahydrochloride as described above. Slides were counter-stained with Mayer's hematoxylin. Negative controls were performed by replacing VLPs with PBS.

Bacterial strains

The *H. pylori* strains 17875/Leb, 17875babA1::kan babA2::cam, and J99 were obtained from the Department of Medical Biochemistry and Biophysics, Umeå University, Sweden (Mahdavi et al. 2002). The 17875/Leb strain is a spontaneous mutant that binds Le^b but does not bind to sialylated antigens (Mahdavi et al. 2002). *H. pylori* 094UK, 097UK, 101UK, 111UK, 123UK, 127UK, 131UK, and BO265 were obtained from the Department of Molecular Biology, Max-Planck Institut für Infektionsbiologie, Berlin, Germany.

Bacteria were grown in Pylori agar (BioMérieux, Marcy l'Étoile, France) at 37°C under microaerobic conditions. For strain 17875babA1::kan babA2::cam, culture media included also 20 mg/L Chloramphenicol (Sigma) and 25 mg/L Kanamycin (Sigma).

Immunoblot for BabA and SabA adhesins

Bacteria were collected from plates using 1 mL PBS and centrifuged at 2400 g for 5 min; proteins were extracted with the lysis buffer RIPA containing 1 mM PMSF, 1 mM Na₃VO₄, and protease inhibitor cocktail (Roche, Germany). Protein concentration was determined using the BCA protein assay kit and 50 µg was loaded in acrylamide gel (stacking 5%/resolving 10%) for electrophoresis. Proteins were transferred to immunoblot polyvinylidene difluoride membranes (Hybond-P PVDF Membrane, Amersham Biosciences) at 50 V for 1 h. Membranes were blocked for 1 h in PBS-Tween containing 5% nonfat dried milk prior to incubation overnight at 4°C with BabA (AK277 1:10000) and SabA (AK278 1:5000) antibodies. Blots were incubated with a HRP-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) and finally developed with Immuno-StarTM HRP Chemiluminescent kit (Bio-Rad, CA).

DNA extraction and analysis of babA expression by PCR

Total DNA was extracted from *H. pylori* and PCR was performed, as previously described with forward primer babA_F1 (5'-ATCGGTGAAGCCGCTCAA-3') and equimolar amounts of reverse primers babA_R1 (5'-TTACCCC-CGCAATTGCG-3') and babA_R2 (5'-TTACCGCCGC-ATTGCG-3'). The amplified product has 290 bp (Azevedo et al. 2008).

In vitro binding assay of H. pylori to mice gastric mucosa

H. pylori was labeled with FITC and concentration adjusted as previously described (Falk et al. 1993). Adhesion assays were performed as previously described (Falk et al. 1993). Briefly, paraffin-embedded mice gastric sections were deparaffinized and rehydrated followed by incubation for 1 h with blocking buffer: 1% BSA in PBS containing 0.05% Tween 20. BSA was previously submitted to Periodate oxidation to destroy competitive carbohydrate receptors for *H. pylori* binding (Aspholm et al. 2006). The FITC-labeled bacterial suspension was diluted

5-fold in the blocking buffer and 100 µL were used to incubate each section for 2 h at room temperature. Slides were subsequently washed three times with PBS containing 0.05% Tween 20 and stained with DAPI. Evaluation of bacterial binding was estimated by the number of adhered bacteria under 400× magnification. Each value represents the mean of at least five different fields quantified using the ImageJ software, with significance evaluated with the Student's *t*-test. Whenever referred in the text *H. pylori* adhesion was evaluated separately in the foveolar and glandular regions of mice stomach. The foveolar region was considered as the superficial region of the gastric mucosa including the surface mucous cells, while the glandular region corresponds to the deeper portion of the gastric mucosa including the mucosal glands.

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Conflict of interest statement

None declared.

Abbreviations

BabA, blood group antigen binding adhesin; DVLP, Dijon virus-like particle; Fut2, alpha(1,2)fucosyltransferase gene; *H. pylori*, *Helicobacter pylori*; MAL-I, *Maackia amurensis* lectin I; NVLP, Norwalk virus-like particle; PAS, periodic acid Schiff; SabA, sialic-acid binding adhesin; SNA, *Sambucus nigra* lectin; UEA-I, *Ulex europaeus* lectin I; VLPs, virus-like particles.

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