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Host-dependent Lewis (Le) antigen expression in Helicobacter pylori cells recovered from Leβ-transgenic mice

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Variation of surface antigen expression is a mechanism used by microbes to adapt to and persist within their host habitats. Helicobacter pylori, a persistent bacterial colonizer of the human stomach, can alter its surface Lewis (Le) antigen expression. We examined H. pylori colonization in mice to test the hypothesis that host phenotype selects for H. pylori (Le) phenotypes. When wild-type and Leβ-expressing transgenic FVB/N mice were challenged with H. pylori strain HP1, expressing Leα and Leε, we found that bacterial populations recovered after 8 mo from Leβ-transgenic, but not wild-type, mice expressed Leβ. Changes in Le phenotype were linked to variation of a putative galactosyltransferase gene (β-(1,3)galT); mutagenesis and complementation revealed its essential role in type I antigen expression. These studies indicate that H. pylori evolves to resemble the host’s gastric Le phenotype, and reveal a bacterial genetic locus that is subject to host-driven selection pressure.

For microbes that are obligatory parasites of outbred host species, an important challenge is to adapt to each new individual host (Moxon et al., 1994; Falk et al., 2000; Bayliss et al., 2004; Blaser and Kirschner, 2007). Such co-evolved bacteria use multiple strategies, including stealth, variation, and antidefense (Monack et al., 2004; Blaser and Kirschner, 2007). One mechanism to generate variation is the use of contingency genes to change expression of bacterial cell-surface structures relevant to the hosts being colonized (Moxon et al., 1994; Bayliss et al., 2001; Bayliss et al., 2004).

Humans are polymorphic for the expression of the fucosylated Lewis (Le) antigens on erythrocytes and in other body compartments, including the gastric epithelium (Sakamoto et al., 1989). Helicobacter pylori, the dominant human gastric bacteria (Bik et al., 2006; Andersson et al., 2008), are also polymorphic for expression of Le antigens (Fig. S1; Wang et al., 1999). Most strains predominately express Leα and Leε (type II antigens), which are major human gastric antigens (Simoons-Smit et al., 1996), whereas <5% express Leα and Leβ (type I antigens; Wirth et al., 1996), which are also expressed in the stomach (Sakamoto et al., 1989). H. pylori may vary type II Le expression using a variety of genetic mechanisms (Appelmelk et al., 1998; Wang et al., 1999; Wirth et al., 2006; Sanabria-Valentín et al., 2007; Nilsson et al., 2008).

We have hypothesized that H. pylori Le expression reflects host selection operating on a population of stochastically varying strains that have differential fitness in particular hosts (Webb and Blaser, 2002). Observations in humans naturally colonized with H. pylori (Wirth et al., 1997) and in rhesus monkeys experimentally infected with H. pylori (Wirth et al., 2006) support this hypothesis. However, these studies are not conclusive, because the human studies were correlative, and the monkey studies were an experimental challenge with multiple strains and a small number of study animals (Wirth et al., 1997; Wirth et al., 2006).
Wild-type mice do not express Le\textsuperscript{b} in their stomach. The creation of transgenic mice that express a human \(\alpha\)-1,3/4 fucosyltransferase (accession no. EC 2.4.1.65 from the IntEnz database, available at http://www.ebi.ac.uk/intenz/index.jsp) in their mucus-producing gastric pit cells led to Le\textsuperscript{b} expression (Falk et al., 1995; Guruge et al., 1998). The presence of Le\textsuperscript{b} in the gastric mucosa of these mice and its absence in their nontransgenic littermates presented an opportunity to examine whether host phenotype selects for \textit{H. pylori} phenotypic (Le antigen) expression. We hypothesized that among \textit{H. pylori} strains introduced into “humanized” Le\textsuperscript{b}-transgenic mice but not their isogenic Le\textsuperscript{b}-negative (wild-type) littermates, there would be selection for bacterial Le\textsuperscript{b} expression. In the present study, we verify this hypothesis, and characterize the genetic loci and mechanisms responsible for the changed \textit{H. pylori} phenotype.

RESULTS

\textit{H. pylori} colonization of wild-type and Le\textsuperscript{b}-transgenic FVB/N mice

Conventionally raised, specific pathogen-free transgenic FVB/N Le\textsuperscript{b} mice (\(n = 20\)) and wild-type littermates (\(n = 20\)) were group housed in microisolator cages, maintained on a standard chow diet, and challenged at 6 wk of age (Fig. S2) with \textit{H. pylori} strain HP1 (98–964), which expressed both Le\textsuperscript{x} and Le\textsuperscript{y}, but had no detectable Le\textsuperscript{a} or Le\textsuperscript{b} expression. At varying times after infection, mice of each genotype were sacrificed, the stomachs were homogenized, and \textit{H. pylori} was cultured by serial dilution on selective media. \textit{H. pylori} colonization could only be detected in three out of the five mice in each group after 4 wk but was detected in each mouse at all subsequent time points (8, 16, and 32 wk; \(n = 5\) animals/group/time point). There were no significant differences in bacterial density observed according to mouse genotype at any of the time points surveyed (Fig. 1). In total, we achieved stable (\(10^3\) CFU/mouse stomach), long-term (28 mo) \textit{H. pylori} colonization in both wild-type and Le\textsuperscript{b}-transgenic mice (Fig. 1).

Host-dependent Le antigen expression in mice

We next addressed whether colonization of the wild-type and Le\textsuperscript{b}-transgenic mice would select for differing \textit{H. pylori} Le phenotypes, as determined by Le-specific ELISAs. All bacterial populations recovered expressed Le\textsuperscript{x} and Le\textsuperscript{y}, and there were no significant differences in Le\textsuperscript{x} or Le\textsuperscript{y} expression between sweeps recovered from wild-type or Le\textsuperscript{b}-transgenic mice in either the early or late isolates (Fig. S3). None of the isolates recovered from the wild-type or transgenic mice before the 8-mo time point expressed Le\textsuperscript{a} or Le\textsuperscript{b} (\(n = 10\) assayed/time point), nor were these epitopes detectable in \textit{H. pylori} sweeps recovered from wild-type mice 8 mo after challenge (Table I). Although the ratio of Le\textsuperscript{x} to Le\textsuperscript{y} in the \textit{H. pylori} cells recovered from the 8-mo wild-type mice was similar to the inoculum strain, their overall expression was higher. However, in \textit{H. pylori} sweeps from the 8-mo Le\textsuperscript{b}-transgenic mice, there was expression of Le\textsuperscript{b} in addition to Le\textsuperscript{x} and Le\textsuperscript{y} in four out of the five mice (Table I), which was significantly (\(P = 0.001\)) different from the wild-type mice. In addition, overall Le\textsuperscript{a} expression in the bacterial populations isolated from the Le\textsuperscript{b}-transgenic mice was significantly (\(P = 0.014\)) lower than in populations from the wild-type mice. There was a trend toward higher colonization levels in the four mice carrying Le\textsuperscript{b}-positive \textit{H. pylori} compared with colonization densities of the five wild-type mice (\(P = 0.054\)). This result could reflect increased adhesion to host-expressed Le\textsuperscript{b} through binding by the bacterial Le\textsuperscript{b} ligand, BabA (Ilver et al., 1998). Although this result suggests that bacterial Le\textsuperscript{b} expression enhanced colonization and, thus, may have been positively selected, future studies will be required to confirm this observation.

Mouse humoral responses to \textit{H. pylori} challenge

One hypothesis to explain the drop in Le\textsuperscript{x} expression in the 8-mo Le\textsuperscript{b}-transgenic mice is that an increase in anti-Le\textsuperscript{x} antibodies in the transgenic mice selected for cells with reduced Le\textsuperscript{x} expression, resulting in the emergence of Le\textsuperscript{b} expression, paralleling phenomena that have been observed previously in other organisms (Bayliss et al., 2001). To test this hypothesis, we determined mouse antibody levels against both \textit{H. pylori} whole cells and purified \textit{H. pylori} LPS with distinct Le phenotypes. ELISAs testing serum responses to 98–964 (HP1) whole-cell antigen showed that both wild-type and Le\textsuperscript{b}-transgenic mice progressively developed anti–\textit{H. pylori} IgG responses during the course of the challenge (Fig. S4). No IgG was detected in the uninfected (control) mice, but an IgG response appeared during the early infection period (4–8 wk), rising to the highest levels during late infection (4–8 mo).

Figure 1. Quantitative \textit{H. pylori} culture of wild-type and Le\textsuperscript{b}-transgenic FVB/N mice after experimental challenge with strain HP1. In this single experimental challenge, CFUs were determined by homogenization of a segment of the mouse stomach in sterile PBS (pH 7.4), followed by serial dilution on \textit{H. pylori}-selective medium. At 4 wk, \textit{H. pylori} was not detected in four mice; the means shown are the means for the \textit{H. pylori}-positive mice. \textit{H. pylori} was cultured from the stomachs of all of the other 36 mice in this study. Each of the 40 mice received an independent challenge with the stock culture of strain HP1 (\(n = 5\) animals/group/time point). There were no significant differences in colonization levels between transgenic and nontransgenic animals at any of the time points as determined by a \(t\) test.
There were no significant differences in IgG levels between the wild-type and Le<sup>b</sup>-transgenic mice (n = 10 mice/genotype/time point). These data confirm that the experimental H. pylori challenges induced adaptive humoral responses, as expected. The lack of difference between the groups provides evidence that unlike in previous studies (Guruge et al., 1998), the host genotype did not substantially affect the responses to the heterologous H. pylori antigens.

Next, we tested mouse sera with LPS preparations from wild-type strain J166 (OD values: Le<sup>x</sup> = 0 and Le<sup>y</sup> = 3.36) with strong Le<sup>x</sup> expression, J166 ΔfutC (OD values: Le<sup>x</sup> = 2.22 and Le<sup>y</sup> = 0) with strong Le<sup>y</sup> expression, and J166 ΔfutA/ΔfutB (OD values: Le<sup>x</sup> = 0 and Le<sup>y</sup> = 0) with neither Le<sup>x</sup> nor Le<sup>y</sup> expression. Thus, we could examine the serologic response to specific Le epitopes. First, we examined responses to J166 whole-cell antigen, expecting that there would be robust responses, as observed with 98-964 whole-cell antigen (Fig. S4). The high antibody levels in 4- and 8-mo wild-type (OD value = 1.69 ± 1.48) and Le<sup>b</sup>-transgenic mice (OD value = 1.89 ± 0.98; Table S1) confirmed that the mice were capable of responding to H. pylori antigens and showed no significant difference in response according to mouse genotype.

The responses to the LPS antigens were heterogeneous (Table S1), with a bimodal distribution, and roughly correlated with those to whole-cell antigen. The responses to Le<sup>x</sup> LPS (wild type) and Le<sup>y</sup> LPS (ΔfutA/ΔfutB) were significantly (P = 0.035 and P = 0.011, respectively) higher in the 8-mo compared with the 4-mo mice, and trended in that direction (P = 0.15) for the Le<sup>x</sup> LPS (ΔfutC). Response to LPS also appeared to be independent of LPS Le phenotype; mice with strong anti-LPS responses showed strong responses to LPS of all three Le phenotypes. There were no significant differences in anti-LPS responses between wild-type and Le<sup>b</sup>-transgenic mice. Finally, we asked whether there was an association between anti-LPS response and the H. pylori colonization density in the mice. Comparing the log<sub>10</sub> CFU (2.43 ± 0.98) of the three mice with the highest anti-Le<sup>x</sup> (J166 ΔfutC LPS) responses (3.49 ± 0.7) to the log<sub>10</sub> CFU (2.61 ± 0.92) of those with the lowest responses (0.03 ± 0.001) showed no significant differences; results were parallel for the other LPS preparations (unpublished data).

**Table I.** Bacterial counts and Le antigen phenotypes of H. pylori gastric sweeps from wild-type and Le<sup>b</sup>-transgenic FVB/N mice recovered after 8 mo

<table>
<thead>
<tr>
<th>Source of H. pylori</th>
<th>Sweep designation</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; CFU/stomach&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Le antigen phenotype&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum strain HP1</td>
<td>-</td>
<td>-</td>
<td>Le&lt;sup&gt;x&lt;/sup&gt; Le&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild-type mice</td>
<td>00-4</td>
<td>1.95</td>
<td>0.01 0.01</td>
</tr>
<tr>
<td></td>
<td>00-6</td>
<td>1.86</td>
<td>0.01 2.9</td>
</tr>
<tr>
<td></td>
<td>00-8c</td>
<td>2.43</td>
<td>0.01 2.26</td>
</tr>
<tr>
<td></td>
<td>00-10</td>
<td>2.06</td>
<td>0.01 1.94</td>
</tr>
<tr>
<td></td>
<td>00-12</td>
<td>1.78</td>
<td>0.01 1.73</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.02 ± 0.25</td>
<td>0 ± 0</td>
<td>2.14 ± 0.47 1.49 ± 0.58</td>
</tr>
<tr>
<td>Leb-transgenic mice</td>
<td>00-14</td>
<td>3.68</td>
<td>0.02 0.57 0.86</td>
</tr>
<tr>
<td></td>
<td>00-16c</td>
<td>4.28</td>
<td>0.01 0.15 1.44</td>
</tr>
<tr>
<td></td>
<td>00-18</td>
<td>3.56</td>
<td>0.01 0.46 0.52</td>
</tr>
<tr>
<td></td>
<td>00-20</td>
<td>2.1</td>
<td>0.01 1.88</td>
</tr>
<tr>
<td></td>
<td>00-22</td>
<td>0.78</td>
<td>0.01 0.19</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.88 ± 1.42</td>
<td>0.01 ± 0.27</td>
<td>0.98 ± 0.69 1.4 ± 0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bacterial counts (CFUs) were determined by homogenization of one third of the mouse stomach in sterile PBS (pH 7.4), followed by serial dilution on selective medium. The mean bacterial counts recovered from wild-type and transgenic mice were not significantly different.

<sup>b</sup>Phenotype was determined by standardized ELISA using monoclonal antibodies to Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, or Le<sup>f</sup>, with values expressed as OD. ELISAs were performed in triplicate wells for each strain, and the OD values presented represent the mean of three independent experiments. An OD value ≥0.1, indicated in bold, is considered significant.

<sup>c</sup>Sweeps of H. pylori gastric isolates that were selected as sources for subculture to examine phenotypes of isolated colonies (Fig. 2).

<sup>d</sup>Significantly different than for populations isolated from wild-type mice (P = 0.001).

<sup>e</sup>Significantly different than for populations isolated from wild-type mice (P = 0.014).

**Analysis of babA in mouse-derived H. pylori isolates**

One hypothesis to explain why colonization densities were higher in the Le<sup>b</sup>-transgenic mice carrying Le<sup>b+</sup> H. pylori could be the increased bacterial adherence to the gastric epithelia by the H. pylori Le<sup>b</sup> ligand, BabA. In a previous challenge of rhesus monkeys (Solnick et al., 2004), a gene conversion event occurred that replaced a portion of babA with babB (as demonstrated by a series of PCRs), which resulted in a loss of Le<sup>b</sup> adhesion. To examine this question, we first sought to confirm the previous findings (Solnick et al., 2004) by studying a strain (J166) that successfully colonized rhesus monkeys and comparing it with a J166-derived strain recovered from a monkey 10 mo later (98-169) from our previous monkey challenge studies (Wirth et al., 2006). Sequence analysis of the band produced by primers 834F and
AR6 amplified in 98-169 show that the middle region of babA was replaced by babB, indicating that gene replacement had occurred, providing independent confirmation of the previous findings in monkeys (Fig. S7; Solnick et al., 2004). On this basis, we turned to the mouse isolates. When we tested the inoculum strain 98-964 and one isolate each from the wild-type (03-261) and Leb-transgenic mice (03-270), we found that the babA allele was intact in all cases (Fig. S7), supporting the hypothesis that there was no selection away from babA, and that its maintenance is important for mouse colonization, differentiating the findings in monkeys and mice.

**Phenotypic diversity of single-colony H. pylori isolates**

To analyze the diversity in Le expression within H. pylori populations recovered from the wild-type and transgenic mice 8 mo after challenge, single colonies were isolated from sweeps 00-8 (from a wild-type FVB/N mouse) and 00-16 (from an Leb-transgenic mouse; Fig. S2). The single colonies isolated from sweep 00-8 only showed expression of Lea and Leb, with no detectable Lea or Leb expression, confirming results obtained from the gastric sweeps (unpublished data). In contrast, 9 out of 11 single colonies isolated from the 00-16 sweep expressed Lea and/or Leb in addition to Lea and Leb (Fig. 2 A). The phenotypic diversity within the bacterial populations derived from the gastric sweeps of these mouse stomachs is similar to that reported in humans (Wirth et al., 1999; González-Valencia et al., 2008). Expression of Lea in five of the 00-16 single colonies, in addition to Leb, indicates diminished futC activity in these isolates (Sanabria-Valentín et al., 2007; Nilsson et al., 2008), preventing substrate Lea from being fucosylated to form Leb (Fig. S1). The ratios of Lea to Leb and Lea to Leb expression in these single colonies are strongly correlated (R = 0.89; P < 0.001; Fig. 2 B), indicating that futC is governing the relative expression levels of the mono- and difucosylated antigens in tandem for the type I and II pathways (Fig. S1). From these studies, three strains were chosen for further genotypic and phenotypic analysis: HP1 (98-964, the inoculum strain from which Leb was not detectable), 00-8B (03-261, a non-Leb expressor isolated from a wild-type mouse), and 00-16A (03-270, an Leb expressor isolated from an Leb-transgenic mouse; Table I).

**DNA sequence analysis of the Le antigen synthesis genes**

Nucleotide sequence analysis was performed to determine the genetic basis for the phenotypic differences in Le antigen expression in the selected representative mouse-derived H. pylori strains. A β-(1,4) galactosyltransferase involved in Lex and Ley synthesis is encoded by galT (Fig. S1), which does not contain polynucleotide repeat regions; however, the upstream intergenic region varies in length between strains (not depicted). Because sequence analysis showed that all three strains share an identical upstream region (unpublished data), their different Le antigen phenotypes were not attributed to galT locus variation.

Sequence analysis of the α-(1,3/4) fucosyltransferase gene (futA; Fig. S1), which affects both the type I and II Le synthesis pathways, showed that all three strains had identical in-frame futA alleles. Sequence analysis of futB (Fig. S1) revealed extensive intrastrain poly-C tract length variation among the three strains (Fig. S5), but overall there were no significant differences between the three strain populations. Thus, differential activity of the α-(1,3/4) fucosyltransferases in these strains does not correlate with the Leb phenotypic differences observed but is consistent with the overall phenotypic variation in Le expression within the populations of cells, as reflected in the Le phenotypes of single-colony isolates (Fig. 2 A).

The poly-C tract of futC was cloned and sequenced for each strain (10 clones/strain) and, as expected, revealed

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Le phenotypes of H. pylori single colonies isolated from wild-type and Leb-transgenic FVB/N mice recovered after 8 mo. (A) 11 colonies, each independently picked, were isolated from H. pylori gastric sweep 00-16, recovered 8 mo after challenge from Leb-transgenic mouse 16 (Table I), and studied. Standardized ELISAs, performed three independent times using monoclonal antibodies to Lea, Leb, Lea, or Leb, were performed in triplicate for each isolate, and the OD values presented from one representative experiment are shown (means ± SD). OD values ≥0.1 are considered significant. (B) Log10 Le mono- and difucosylated antigen expression ratios of 00-16-derived H. pylori strains (single colonies were picked and expanded for assay).
extensive intrastrain variation (Sanabria-Valentín et al., 2007). The primary sequence data suggest that only 2 (61C6 and 70C7) out of 30 intrastrain variants tested would produce a full-length α-(1,2) fucosyltransferase (Fig. S6 B). Although multiple mechanisms can be present (Wang et al., 1999), some α-(1,2) fucosyltransferase activity must exist in strains 98-964, 03-261, and 03-270, because all three produce Leb and 03-270 also expresses Leb. The distribution of poly-C tract lengths in Leb-expressing 03-270-derived clones was significantly (P < 0.01 and P < 0.001) different from that in clones derived from 98-964 and 03-261, respectively, consistent with enhanced selection for Leb expression in the Leb-transgenic mice.

### Nucleotide sequence analysis of β-(1,3)galT

Sequence analysis of β-(1,3)galT showed that the non-Leb-expressing strains 98-964 and 03-261 were identical in sequence (Fig. 3 A). Both contain C14 poly-C tracts, whereas Leb-expressing strain 03-270 has a C16 tract (Fig. 3 B). Based on the predicted (ATG) start codon of β-(1,3)galT in sequenced strain J99 (Fig. 3 A; Alm et al., 1999) as the putative translation start site, all three strains appear to have an out-of-frame β-(1,3)galT (Fig. 3 D). Thus, primary sequence data would not be sufficient to explain differences in Le antigen expression between these strains. However, if the alternate translation initiation codon (TTG), active in ~9% of H. pylori genes (Alm et al., 1999), is used in these strains at the same position as the GTG start codon (~8% of H. pylori genes) of β-(1,3)galT in reference strain 26695 (Fig. 3 A; Tomb et al., 1997), then frame status is determined by poly-C tract length. Strains 98-964 and 03-261 encode seven β-(1,3)galT heptad repeats versus four in strain 03-270 (Fig. 3 C); similar changes were observed in a previous mouse gastric challenge (Salain et al., 2005).

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**Figure 3.** Nucleotide sequence alignments of informative β-(1,3)galT regions in three H. pylori reference strains and in three studied strains related to mouse infection. (A) For H. pylori reference strains J99, HPAG1, and 26695, and for the mouse-derived H. pylori strains 98-964 (inoculum), 03-261 (recovered from a wild-type mouse), and 03-270 (recovered from an Leb-transgenic mouse), the 5′ regions including the putative translational initiation codons of β-(1,3)galT are boxed in blue. An alternative TTG start site (boxed in green and present in 8.1% of 26695 ORFs) aligned with the H. pylori 26695 β-(1,3)galT GTG start site (present in 9.7% of 26695 ORFs). (B) Homocytosine tract region, boxed in orange. (C) 21-bp repeat region, encoding KYDELTG repeats. (D) Predicted amino acid sequence of the 5′ end of β-(1,3)galT. Compared with strain J99, the H. pylori strains obtained from these mouse experiments contain a 5-bp deletion resulting in premature termination (red asterisks). The β-(1,3)galT sequence for strain 03-261 was identical to strain 98-964 (not depicted).
Mutagenesis of β-(1,3)galT

Because of these uncertainties, allelic replacement mutagenesis was performed to determine whether β-(1,3)galT is essential for *H. pylori* type I Le antigen expression (Fig. S8 B). A plasmid containing a copy of β-(1,3)galT interrupted by a nonpolar kanamycin resistance (*aphA-3*) cassette (p98B13k) was introduced into test *H. pylori* strains via natural transformation (Fig. S8 B), and insertion of the plasmid-encoded sequences was confirmed by PCR analysis of chromosomal DNA from transformed cells. Strains 99-8 and JP26 were included as positive controls for Lea and Leb phenotypic expression, respectively, and strain JP26 was transformed with pCTB8 (Cover et al., 1994) as a positive control for transformation. Introduction of p98B13k resulted in loss of Leb expression in strains JP26 and 03-270 (Fig. 4 A), and loss of Lea expression in strain 99-8 (not depicted). Also as expected, introduction of control plasmid pCTB8 into JP26 had no effect on Le antigen phenotype (unpublished data). In total, these studies provide evidence that open reading frame (ORF) *jhp0563* encoding the presumed β-(1,3)galT is essential for synthesis of type I Le antigens.

Complementation of β-(1,3)galT null mutants

To determine whether the loss of type I Le antigen expression was specifically caused by β-(1,3)galT inactivation and not an adventitious event, complementation studies were performed. To accomplish this, *jhp0563* from strain 03-270 was introduced in trans (p70B13comp) into *H. pylori* strains in which the native ORF had been interrupted (Fig. S8 C), and its placement was confirmed by PCR. Introduction of β-(1,3)galT at the ureA locus resulted in restoration of type I Le antigen expression in the JP26 and 03-270 β-(1,3)galT null mutants (Fig. 4 A). Complementation of strain 03-261 led to a low level but new expression of Leb, which had not been observed in either the wild-type or mutant strains (Fig. 4 A). However, there was no complementation of Lea expression in the 99-8 β-(1,3)galT null mutant and no de novo synthesis of Lea or Leb in strain 98-964 (unpublished data). Collectively, these results provide evidence that β-(1,3)galT is necessary, but not sufficient, for type I Le antigen expression.

Sequence analysis of complemented β-(1,3)galT

Because colonies of *H. pylori* represent a mixture of cells of varying genotypes (Appelmelk et al., 1998; Kuipers et al., 2000; Sanabria-Valentin et al., 2007), especially in reference to metastable loci such as homopolymeric tracts (Salain et al., 2004; Sanabria-Valentin et al., 2007), we analyzed nucleotide sequences from the complemented strains. Analysis of the 5′ 500 nucleotides of the complemented copy of β-(1,3)galT revealed sequences identical to 03-270 wild-type β-(1,3)galT in all five complemented mutants tested. These results indicate that sequence variation in the 5′ region is not responsible for the phenotypic variation observed. However, as determined by direct PCR sequencing, poly-C tract lengths varied among the complemented mutant strains. Therefore, Le antigen phenotypes were determined by ELISA for 10 single colonies isolated from strain 03-270::p98B13k::p70B13comp. The Lea expression in these strains ranged from the level of 03-270 wild type to no detectable expression (Fig. 4 B). The β-(1,3)galT heptad repeat region was identical in length in these single-colony isolates, indicating that variation in Lea expression is not caused by variation in this region (unpublished data). To identify the basis of this variation, we examined genomic DNA from isolate 70C1A with high Leb expression (Leb = 0.74), from isolate 70C1E with an intermediate Leb phenotype (Leb = 0.25), and from isolate 70C1F with no detectable Leb expression to PCR amplify the poly-C tract region (OD values are shown). After cloning the products, sequence analysis of the recombinant pGEM-T Easy plasmids revealed extensive β-(1,3)galT poly-C tract length variation (Fig. 4 C). 4 out of 10 70C1A-based sequences contained C16 poly-C tracts, identical to 03-270 wild type, versus 2 out of 10 from the 70C1E sequences and 0 out 10 from the 70C1F sequences. The number of clones with a β-(1,3)galT poly-C tract, identical to the 03-270 poly-C tract, correlated with the level of Leb expression in each isolate. The 70C1A poly-C tract lengths (16.2 ± 1.48 nucleotides) and the 70C1E poly-C tract lengths (15.8 ± 2.04 nucleotides) were significantly (P < 0.001) longer than those in 70C1F (11.8 ± 0.79 nucleotides). Thus, within populations of *H. pylori* cells, β-(1,3)galT frame status as well as homopolymeric tract length vary extensively, critically affecting Leb expression.

Flow cytometric analysis of *H. pylori* β-(1,3)galT mutants

To better understand the distribution of Leb phenotypic expression within a population of *H. pylori* cells, wild-type strains and their β-(1,3)galT mutants were examined by flow cytometry. Cells were incubated with an anti-Leb monoclonal antibody and anti-*H. pylori* polyclonal antibodies as a positive control. Cells of all three *H. pylori* strains (98–964, 03-261, and 03-270) were detectable with anti-*H. pylori*, essentially to the same extent (Fig. 5 B). As expected, when stained with antibodies directed against Leb, the 03-270 wild-type strain showed the greatest levels of fluorescence, with a bimodal distribution, indicating that most but not all cells were Leb positive (Fig. 5 C). In contrast, also as expected, the 03-270 β-(1,3)galT mutant showed fluorescence levels similar to the background level of cells stained with secondary antibodies alone (Fig. 5 C). The 03-270 β-(1,3)galT–complemented mutant was positive for Leb, but the percentage of Leb-positive cells was lower than in the wild type (Fig. 5, C and F), indicating that the complementation was partial. The net mean fluorescence intensity (MFI; region M1) for the Leb-positive cell populations in the wild-type and complemented strains was similar, indicating that a proportion of the cells had been fully complemented (Fig. 5 G). Similar flow cytometry experiments performed on JP26 and 03-261 indicated that the complemented mutant populations were a mixture of Leb+ and Leb− cells (Fig. 5, D and E). The Leb positivity in the JP26-complemented cells was significantly (P < 0.001) lower than in the wild-type strains, confirming that complementation was partial and less efficient than in
However, as in strain 03-270, net MFI levels showed full complementation in a portion of the population (Fig. 5 G). Collectively, these results paralleled phenotypes determined by ELISA (Fig. 4 A) and define the range of Leb phenotypes exhibited by wild-type *H. pylori* strains and their β-(1,3)galT mutants.

**DISCUSSION**

These studies showed that experimentally challenged wild-type and Leb-transgenic FVB/N mice can stably maintain *H. pylori* gastric colonization for up to 8 mo, regardless of Le antigen expression, consistent with studies in other mouse genetic backgrounds (Takata et al., 2002; Lozniewski et al., 2003). That *H. pylori* populations expressing Leb were somewhat more efficient than non-Leb expressors in colonizing Leb-transgenic mice (Table I) may reflect greater host tolerance to self-antigens, although future studies are needed to confirm this observation. This phenomenon also could be attributed to enhanced adhesion of bacterial cells to the gastric epithelia via BabA binding to host Leb, because unlike in monkeys (Solnick et al., 2004), babA recovered from the mice 8 mo after challenge showed intact copies of babA and no evidence of gene conversion (Fig. S7). The host Leb-bacterial BabA interaction also could be a target of selection. Enhanced bacterial Leb expression could result in BabA-mediated bacterial aggregation, contributing to the higher colonization densities observed in Leb-transgenic mice; further experimentation is required to test this hypothesis.

Selection for Leb expression among *H. pylori* cells persistently colonizing Leb-positive transgenic but not Leb-negative wild-type mice is consistent with increased fitness of *H. pylori* variants that match the Le phenotype of their host, extending previous studies in rhesus monkeys (Wirth et al., 2006) and in some (Wirth et al., 1998; Heneghan et al., 2000) human studies. One hypothesis to explain the appearance of Leb expression in *H. pylori* recovered from the transgenic mice 8 mo after challenge is the development of an anti-Lex response in these mice, which is consistent with the lower expression of Lex in the *H. pylori* cells recovered from the Leb-transgenic mice after 8 mo. Such a response would provide selection for the expansion of Le expression variants, a phenomenon that has been observed previously in *Neisseria meningitidis* (Bayliss et al., 2001). To test this hypothesis, we determined levels of anti-Le antibodies by ELISA using purified LPS with well-defined Le antigen phenotypes. The results showed a wide range in responses among the mice, with no correlation between mouse genotype and antibody response. Further, antibody responses were independent of both LPS Le phenotype and colonization density of the mice. These results...
provide evidence that serum antibody pressure was not the driving force behind the emergence of bacterial Leb expression. However, our observations are limited by a small sample size and an inability to directly test for anti-Lea and anti-Leb antibody levels. Future studies are needed to further investigate this hypothesis.

Sequence analysis showed that selection of Leb-positive H. pylori variants in the transgenic mice was mediated by phase variation of β-(1,3)galT. Solnick et al. (2004) provided evidence that phase variation of balbA occurred in vivo, supporting the hypothesis that this is a gene regulation mechanism used by H. pylori to adapt to changing host environments.

Experimental challenge of wild-type mice and gerbils for up to 5 mo with H. pylori isolates led to no substantial Le antigen expression diversification (Wirth et al., 1999). Similarly, when wild-type FVB/N and Leb-transgenic mice were experimentally challenged for 8 or 16 wk, no change in Le antigen expression was detected (Guruge et al., 1998). One explanation for those results is that there was no phenotypic selection, or alternatively, that the H. pylori founding populations were too small and/or colonization periods too short for detection of differential fitness (Webb and Blaser, 2002). That no changes were observed before 8 mo in the present studies may reflect both the relatively low level of H. pylori colonization of conventionally raised mice compared with humans (Atherton et al., 1996) and/or relatively small differential fitness (Webb and Blaser, 2002) because of bacterial Leb expression in the Leb-transgenic mice.

Figure 5. Leb expression by H. pylori wild-type and β-(1,3)galT mutants. H. pylori cells were incubated with mouse anti-Leb IgM and human anti-H. pylori IgG and detected with fluorescent secondary (2°) antibodies. Irrelevant staining in the absence of anti-Leb or anti-H. pylori are displayed for cells incubated with 2° antibodies alone. (A) Dot plot displaying forward and 90° angle scattered laser light intensities of PI-labeled bacterial cells. Intact bacteria were selected using region R1. For each sample 20,000 R1-gated events were counted (green, 30% log density; pink, 9% log density; blue, 2.7% log density; orange, 0.81% log density). (B) Histograms comparing wild-type H. pylori strain 03-270 (WT), β-(1,3)galT null mutant (Mut), and β-(1,3)galT-complemented mutant (Comp) bacteria labeled with allophycocyanin–anti–H. pylori. (C–E) Histograms of H. pylori strains 03-270 (C), JP26 (D), and 03-261 (E) labeled with FITC–anti-Leb. Region M1 denotes the Leb-positive cell populations, as determined by the crossover point between the wild-type and β-(1,3)galT null mutant plots. (F) Net percentage of Leb-positive cells in β-(1,3)galT mutant strains. Net values were calculated by subtracting the percentage of β-(1,3)galT null mutant cells in region M1 from the percentage of wild-type and complemented cells in M1. There were significantly (*, P < 0.001) fewer Leb-positive cells in the JP26 β-(1,3)galT-complemented mutant cell population than the JP26 wild-type cell population. (G) Net FITC MFI. Values were normalized based on the number of Leb-positive cells, and net values were calculated as in F (means ± SD). C and F represent three independent experiments.
Sequence analysis of β-(1,3)galT in HP1 and its descendants revealed a 5′ 5-bp deletion compared with H. pylori reference strain J99, suggesting either a truncated ORF or that the annotated 5′ ATG is not the initiation codon in these strains. Because Leb is expressed in strain 03-270, a more downstream start codon may be used for translation, translational frameshifting is occurring, or β-(1,3)galT is undergoing recombination with its upstream homologue, jhp0562, in some H. pylori cells within the population. TTG present in some strains at the same position as the 26695 initiation GTG (Fig. 3 A) may be an alternative initiation codon (Alm et al., 1999), representing another translational control locus of Le synthesis gene expression.

Our findings indicate that as with H. pylori futA, futB, and futC, poly-C tract length likely regulates β-(1,3)galT translation, with C_{16} in 03-270 critical for Leb expression. The three strains tested also differed at the β-(1,3)galT heptad repeat region, paralleling observations in another mouse challenge experiment (Saluin et al., 2005), potentially explaining β-(1,3)galT enzymatic activity variation, paralleling that shown in futA and futB (Ge et al., 1997; Lin et al., 2006; Ma et al., 2006; Nilsson et al., 2006).

Although the inoculum strain HP1 expressed Leα and Leβ in relatively equal proportions, the gastric sweeps and single colonies varied greatly in their Le antigen phenotypes. That in relatively equal proportions, the gastric sweeps and single colonies have evolved to facilitate Le expression control, particularly types in the complemented strains compared with wild type by ELISA, providing an alternative phenotyping method. Although ELISA determines the overall phenotype of a cell population, flow cytometry determines the phenotype of each individual H. pylori cell, providing an indication of the population structure, which is highly advantageous for studying genetically diverse organisms. The results indicate that the descendants of the complemented strain vary in their Leβ phenotype, and as indicated by the sequence data, generated by phase variation of the metastable poly-C tracts of β-(1,3)galT, futA, futB, and futC.

In conclusion, we have developed a tractable animal model to examine the hypothesis that changes in Le phenotypes are a mechanism used by H. pylori to adapt to specific host milieus. Our studies provide evidence that H. pylori can change Le phenotype to the type I pathway in vivo to match the Le phenotype of its host, extending previous studies in humans and rhesus monkeys (Wirth et al., 1997; Wirth et al., 2006). That the pathway involves phase-variable Le antigen synthesis genes provides a mechanistic explanation for the observed phenotypic changes, and can be harnessed to allow quantitative analysis of the force of selection.

**Materials and Methods**

**Animals.** Wild-type and Leb-transgenic FVB/N mice breeding pairs were provided by the Washington University Animal Facility, and were bred and maintained under specific pathogen-free conditions in microisolators in a barrier facility, as previously described (Falk et al., 1995; Guruge et al., 1998). All experiments using mice were performed using protocols approved by the Animal Studies Committee of Washington University. DNA was extracted from the mouse tails using the QIAamp Tissue Kit (Promega) and used as a template in two PCR reactions to determine the mouse genotype: one with primers specific for α-actin (control; gene amplified in all mice), and one with primers specific for hGH (only present in transgenic animals; Table S2).

**Challenge of mice with H. pylori strain HP1.** Mice were challenged with H. pylori strain HP1, isolated from a Peruvian patient with gastritis (Guruge et al., 1998). Before inoculation, H. pylori strain HP1 was grown for 24 h and harvested in Brucella broth. Cell concentration was adjusted to an OD of 2 (≈6 × 10^8 cells) at 550 nm, and 0.4 ml of cell suspension was used to inoculate, via orogastric gavage, 20 wild-type FVB/N and 20 Leα-transgenic FVB/N mice. Orogastic challenge was repeated for three consecutive days. At 4 wk, 8 wk, 4 mo, and 8 mo, mice of each genotype were sacrificed and their stomachs were removed. One third of each stomach was homogenized in PBS (pH 7.4), and undiluted and 1:10 diluted suspensions were plated on Skirrow’s medium agar plates (BBL Microbiology Systems) and incubated for ≈72 h at 37°C and 5% CO₂. CFUs were determined for each gastric sweep and cell populations were harvested; 10–11 single colonies were picked from each gastric sweep and expanded for further analysis.

**Bacterial strains and growth conditions.** H. pylori strains used in this study were routinely grown on Trypticase soy agar/5% sheep blood plates (BBL Microbiology Systems) or Brucella agar (BA) with 10% newborn calf serum (NCS) supplemented with the appropriate antibiotic (e.g., vancomycin, kanamycin, or chloramphenicol). H. pylori strains JP26, a wild-type Leb-positive strain isolated in Japan, and 99–8, an Leb-positive strain, were included as controls in mutagenesis and complementation experiments.

**Determination of Le antigen phenotypes.** H. pylori Le antigen phenotypes were determined by ELISA using monoclonal antibodies to Leα, Leb, Leα, or Leβ (Signet Laboratories, Inc.) and protocols described previously (Wirth et al., 1996). ODs at 410 nm were determined on a microplate reader (MRX; Dynatech Laboratories Inc.). Corrected OD values were determined by averaging the OD values of two or three wells per sample and subtracting the blank (Escherichia coli strain HB101).

**Determination of anti-H. pylori antibody levels in mice.** Levels of anti-H. pylori antibodies in mouse sera were determined by ELISA, essentially as previously described (Wirth et al., 1997). Mouse sera were tested against H. pylori strains 98-964 and J166 whole-cell antigens, as well as the following...
LPS antigens: wild-type strain J166 (Le^+ = 0; Le^- = 3.36), J166 ΔfutA/ΔfutB (Le^- = 0; Le^- = 0), and J166 ΔfutC (Le^- = 2.22; Le^- = 0; a gift of E. Sanabria-Valentin, New York University School of Medicine, New York, NY). DNA sequence analysis of Le antigen synthesis genes. Three H. pylori isolates were chosen for sequence analysis: HP1 (98-964; inoculum strain), 00-8B (03-261; non-Le^- expressor, isolated from a wild-type mouse), and 00-16A (03-270; Le^- expressor, isolated from an Le^- transgenic mouse). Isolates were grown for 48 h, and harvested in 1 ml sterile PBS. Cells were pelleted for 5 min at ~4,300 g, and the extracts were prepared for genomic analysis with the Wizard Genomic DNA Purification Kit (Promega).

Genomic DNA recovered from these strains was used as a template for PCR amplification of the Le antigen synthesis genes using primers specific for each known gene (Table S2). PCR amplification was confirmed by agarose gel electrophoresis, and products were purified using a PCR purification kit (QIAGEN), quantified, and subjected to sequence analysis (SeqWright, Inc.). Because of the inherent difficulties of analyzing long homonucleotide repeat regions and regions with a high GC content (Stirling, 2003), the poly-C tract regions and regions with a high GC content were amplified with primers specific to these regions (Table S2). PCR products underwent direct sequence analysis or were cloned into pGEM-T Easy (Promega), and recombinant plasmids were analyzed by standard sequencing methods (SeqWright, Inc.) and by using a protocol for GC-rich DNA (GeneWiz).

To further characterize the intrastrain variation in the homonucleotide regions of futB and futC, the pGEM-T Easy plasmids containing PCR fragments of these regions were transformed into competent E. coli DH5α (Invitrogen) and plated on Luria-Bertani agar plates with 50 µg/ml X-gal (Thermo Fisher Scientific) and 100 µg/ml ampicillin. For each strain, ~50 transformants were selected, and the recombinant plasmids were purified and subjected to sequence analysis with the universal primers TF7 and SP6K (Promega). To determine the nucleotide sequence of the 5’ region of the complemented copy of β-(1,3)galT, PCR amplification was performed using primers A17476 and jhp0563(+482)R (Table S2), and products were purified and sequenced as described. To determine the length of the poly-C tract in the complemented mutants, 10 single colonies were isolated from 03-270:p98B13k:p70B13comp and their Le antigen phenotypes were determined. Subsequently, the isolates with the highest, lowest, and intermediate Le^- expression provided template genomic DNA for PCR amplification of the β-(1,3)galT poly-C tract region using primers jhp0563(+351)F and Gal(1,3)R(+770). PCR products were cloned into pGEM-T Easy, recombinant plasmids were transformed into E. coli as described, recombinant plasmids were purified from 10 single transformants per strain, and DNA sequence analysis was performed using primers jhp0563(+351)F and Gal(1,3)R(+770).

Construction of β-(1,3)galT null mutants. To disrupt β-(1,3)galT, a knockout plasmid, p98B13k, was constructed. The β-(1,3)galT ORF was amplified from strain 98-964 (HP1) with primers Jhp0562(+630)F and Jhp0564(-10)R (Table S2), and the 1.8-kb product was cloned into pGEM-T Easy. A nonpolar kanamycin resistance cassette (pGEM-T Easy, recombinant plasmids were transformed into E. coli as described, recombinant plasmids were purified from 10 single transformants per strain, and DNA sequence analysis was performed using primers jhp0563(+351)F and Gal(1,3)R(+770).

Immunofluorescent labeling and flow cytometry. For each H. pylori strain examined by flow cytometry, one plate of cells was harvested and resuspended in 1 ml of sterile saline and pelleted at 8,000 rpm for 5 min, washed, pelleted, and resuspended in 1.2 ml saline. The cell suspension was then passed through a 40-µm cell strainer (Falcon; BD), and 50-µl aliquots were pelleted and resuspended in 100 µl 1% Tween 20 in PBS buffer (pH 7.4) to prevent cell aggregation. Cells were incubated for 30 min with 10 µl of 1:25 diluted anti-Le^- monoclonal antibody (mouse IgM) as described, washed in Tween-PBS buffer to remove unbound antibody, and resuspended in buffer. As a positive control, cells were also incubated for 30 min with 10 µl of 1:25 diluted high titer serum from an H. pylori-positive patient (Blaser, 1995). Cells were washed again, resuspended, and stained for 20 min simultaneously with 10 µl of 20 µg/ml Alexa Fluor 488 goat anti-mouse IgM (μ chain specific) and Alexa Fluor 647 goat anti-human IgG (human specific; Invitrogen). The cells were washed again and resuspended in 100 µl of buffer. Then, 400 µl of 1.25% formaldehyde in saline was added, followed by 5 µl of 0.1 mg/ml propidium iodide (PI). Cells were allowed to fix overnight at 4°C. As controls, cells were reacted with the secondary antibody alone. Flow cytometric analyses were performed on a FACSCalibur (BD). PI fluorescence intensity was used to discriminate cells from inorganic particulates. Forward scatter and 90° angle scattered laser light intensities were used to distinguish intact bacteria (R1) from cellular debris (Fig. 5 A). For each sample, 20,000 R1-gated events were acquired in listmode and subsequently analyzed using CellQuest Pro software (BD).

Statistical analyses. The Welch two-sample t test and the Mann-Whitney test were used where appropriate, with P < 0.05 considered significant. Ratios of Le^- to Le^- expression of 0 for 00-16-derived isolates were adjusted to 0.01 to calculate log10 values in Fig. 2 B.

Online supplemental material. Fig. S1 outlines H. pylori Le antigen synthesis pathways. Fig. S2 is a schematic of the experimental challenge of wild-type and Le^-transgenic mice with H. pylori. Fig. S3 shows the Le^- and Le^- phenotypes of H. pylori populations recovered 4 wk to 8 mo after challenge of wild-type and Le^-transgenic mice. Fig. S4 shows serum levels of anti-H. pylori IgG in mice after challenge with strain HP1 (98-964). Table S1 shows serum levels of anti-LPS antibodies after challenge with strain HP1 (98-964). Fig. S5 shows the variation in serum levels of anti-LPS antibodies after challenge with strain HP1 (98-964). Table S1 shows the complementation strategy for β-(1,3)galT. Table S2 shows serum levels of anti-Le^- antigen IgG in mice 4 or 8 mo after challenge with H. pylori strain HP1. Table S2 lists the oligonucleotide primers used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090683/DC1.
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