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Role of *futC* slipped strand mispairing in *Helicobacter pylori* Lewis^y phase variation

Edgardo Sanabria-Valentín^a, Marie-Teresa C. Colbert^a, and Martin J. Blaser^{a,b,c,*}

^a Department of Microbiology, New York University School of Medicine 550 First Avenue, New York, NY, 10016

^b Department of Medicine, New York University School of Medicine 550 First Avenue, New York, NY, 10016

^c VA Medical Center, 423 E23rd Street, 6026W, New York, NY 10010

Summary

The O antigen of the *Helicobacter pylori* lipopolysaccharide is composed of repeating units of fucosylated Lewis (Le) antigens. The $\alpha(1,2)$ -fucosyltransferase (*futC*) of *H. pylori*, which catalyzes the conversion of Le^x to Le^y by addition of fucose, is subject to slipped-strand mispairing involving a homonucleotide (poly-C) tract. To explore the distribution of Le phenotypes within *H. pylori* cells grown in vitro, 379 single colonies of strain J166 were examined for Le expression. Two major populations with reciprocal Le^x/Le^y phenotypes were identified. Phenotypes correlated with *futC* frame status, suggesting that strain J166 represents a mixed population with respect to *futC* poly-C tract length, which was confirmed by a translational reporter. After hundreds of generations in vitro, phenotypes did not change significantly, indicating that the observed J166 Le diversity reflects the founding population. Since slipped-strand mispairing in the *futC* poly-C tract was postulated to explain the Le^y phenotypic change observed in J166 derivative strain 98-169 isolated 10 months after rhesus monkey challenge, in trans complementation with in-frame *futC* was performed. Le^y synthesis was restored and Le^x expression was reciprocally lowered. From these studies, we confirmed the principal role of *futC* slipped-strand mispairing in Le antigenic variation in vitro and in vivo.

Keywords

Bacterial pathogenesis; *Helicobacter*; Phase variation; Lewis antigens

1. Introduction

Helicobacter pylori are gram-negative, microaerophilic spiral bacteria that colonize the human gastric mucosa and usually persist for the life span of the host [1]. Although more than 50% of the world's population carries *H. pylori*, only a small percent develop clinical consequences [2]. *H. pylori* has a highly plastic genome, which may facilitate its persistence in the human stomach for decades [3,4]. Within individual hosts, *H. pylori* variants arise over relatively short periods (months to years) [5], suggesting that the *H. pylori* genome has evolved to diversify after host colonization [6]. Phase variation, defined as the random, reversible, high frequency

*Corresponding author: Martin J. Blaser, Department of Medicine, New York University School of Medicine, 550 First Avenue, New York, NY 10016, Phone: (212) 263-6394 Fax: (212) 263-3969, e-mail: martin.blaser@med.nyu.edu.

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switch between “all-or-none” phenotypes in an individual cell of a clonal population [7], is one strategy used by *H. pylori* to create phenotypic diversity during colonization of the host [8,9]

The mechanisms used by *H. pylori* to escape host immunity are gradually becoming understood [1,10–13]. The discovery that *H. pylori* produces Lewis (Le) antigens identical to those found in human gastric epithelium suggests that molecular mimicry helps evade host responses [14, 15]. Approximately 85% of *H. pylori* clinical isolates express cell-surface fucosylated oligosaccharide (Le) antigens as the terminal saccharides of their lipopolysaccharide (LPS) molecules [16]. First described on the surface of human erythrocytes, Le antigens are expressed by other human tissues, including gastric epithelial cells [17]. *H. pylori* undergoes Lewis phase variation at high frequencies in vitro (e.g. variation between Le^x and i antigen is in the range of 0.2–0.5% [18]) which can result in the presence of several LPS variants in a bacterial population derived from a single cell; however Le expression passages remain relatively stable during in vitro passage [19], possibly representing the absence of directional selection. Four of the five genes involved in Lewis antigen synthesis contain intragenic homonucleotide tracts, which have been implicated in the observed phase variation [18,20].

In *H. pylori* cultures obtained from clinical subjects, Wirth *et al.* found that the relative proportion of Lewis expression corresponded to the predominant human Lewis phenotype [21]; a similar effect was observed during experimental rhesus monkey challenge with strain J166 [22,23]. Briefly, J166 colonies recovered after a 10-month challenge of rhesus monkeys were studied. The J166 population used in that experiment had Le phenotypes of 0.27 ODU for Le^x and 2.02 ODU for Le^y [23], suggesting that in-frame *futC* was predominant before challenge. Strains recovered from two Le^{a+b+} monkeys (in which Le^x predominates in the gastric mucosa [24,25]) expressed mostly Le^x, whereas strains recovered from two Le^{a-b+} monkeys (Le^y predominates in the gastric mucosa [24,25]) expressed mostly Le^y. A +1 frameshift in the *futC* poly-C tract was associated with the observed loss of Le^y expression in representative colonies [23]. Studying *H. pylori* isolates from two patients taken 9 years apart yielded similar results [26].

To better understand the changes observed in the strain J166 population during the rhesus challenge, we sought to characterize the Lewis phenotypes and genotypes present in an in vitro passaged population of J166 cells. Since this strain expresses both Le^x and Le^y (Fig. 2), we hypothesized that it is composed of a mixed population of cells of different phenotypes that together account for the overall observed phenotype. We also wished to examine whether the observed slipped-strand mispairing in the *futC* poly-C tract in the isolates from the monkey experimental challenge is sufficient to explain the Le phenotype. Since the +1 frameshift in the poly-C tract of *futC* was observed in the recovered monkey strains that had lost Le^y expression, we hypothesized that in trans complementation with in-frame *futC* would restore the Le^y phenotype. Our studies demonstrate the central role of the poly-C tract in *futC* in the balance of Le^x and Le^y phenotypes of *H. pylori*, and the role of slipped-strand mispairing of this poly-C tract in the intra-host diversity observed [19], and in the bacterial adaptation to the host [23].

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Strain J166 (Table 1)(s1bm1 *vacA*, *cagA*+, *iceA1* genotype) was isolated as a sweep of colonies from a homogenized diluted human gastric biopsy from a Nashville patient, and has had minimal in-vitro passage (R.M. Peek, Jr., personal communication). Strain 98-169 is a J166 derivative, isolated from rhesus monkey 85D08, and confirmed to have an out-of-frame *futC* [22,23]. All isolates were grown on Trypticase soy agar/5% sheep blood plates (TSA blood

plates) (BBL Microbiology Systems, Cockeysville, MD) or Brucella Agar (BBL) supplemented with 10% Neonatal Calf Serum (BA+10%NCS) (Invitrogen, Carlsbad, CA) and antibiotics as indicated for selection, and incubated at 37°C in an atmosphere of 90% N₂, 5% O₂, and 5% CO₂. XL1-Blue competent *Escherichia coli* cells (Stratagene, La Jolla, CA) were used for cloning purposes and grown on LB media (Qbiogene, Morgan Irvine, CA) supplemented when needed with 100µg/mL of carbenicillin (Sigma).

2.2 Preparation and selection of J166 single colonies

The J166 culture obtained from the Nashville patient was cultured for 72h and cells collected and resuspended in PBS (Cellgro, Herndon, VA), then serially diluted, and inoculated to fresh media. Cultures were allowed to grow for at least 3–5 days, and single colonies were picked and expanded onto new plates and allowed to grow for 3 days. These cells were collected and aliquots prepared for (ELISA) immunoassays, and DNA preparations, and for freezer stocks (Brucella broth + 15% glycerol). For the ELISAs and DNA preparation, cells were washed twice in 0.15M sterile saline. Aliquots then were resuspended in 100µl of saline with 15% glycerol for ELISA, and in 560µl of TE buffer with 30µl of 10% SDS, for DNA preparations which followed standard CTAB/chloroform methods [27].

2.3 Lewis antigen determination

Monoclonal antibodies BG7 or BG8 (Signet Laboratories, Inc., Dedham, MA) with specificity for Le^x or Le^y antigen, respectively, were diluted 1:500 and 1:1000, respectively, in 100µL PBS-1%BSA, and used for ELISA as described [28]. Optical density units (ODU) at 410nm were determined on a microplate reader (MRX, Dynatech Laboratories Inc., Chantilly, VA), and transformed corrected mean optical density (TCMOs) values were calculated for all samples by averaging ODUs for two wells per sample, subtracting the blank (*E. coli* strain HB101), and normalizing based on the J166 parental strain.

2.4 Glycosyltransferase genotyping of *H. pylori* isolates

Genetic analyses of representative J166 single colonies of specified Lewis phenotypes, and of the different mutants and complemented strains was based on sequences of galactosyl- and fucosyltransferases, as reported for genomic strains J99 and 26695. PCR to genotype the *futC* poly-C tract was performed using primers based on the J166 sequence that would amplify ~100bp including the poly-C tract (Table S1). Primer extension sequencing was performed by GENEWIZ, Inc (North Brunswick, NJ). DNA samples were processed with the GENEWIZ GC-rich sequencing protocol (proprietary) and sequence data was collected on an ABI3730xl DNA Analyzer (Foster City, CA). For each segment, forward and reverse sequences were determined, with sufficient replicates to ensure accuracy. Sequence data was analyzed using the ClustalX1.83 software. Genbank accession numbers for J166 and 98-169 *futC* are EF452502 and EF452503, respectively.

2.5 pUC18 frameshift blue/white screening

To determine the proportion of frameshifts occurring in the *futC* poly-C tract in strain J166 and derivative strains MC6 and MC46, a blue-white screen utilizing a *lacZ* translational reporter was developed. Using primers J166fCpCF1EcoRI and polyC(futC)XbaIR (Table S1), an ~80 bp fragment containing the *futC* poly-C tract was PCR-amplified from genomic DNA of several strains of interest. Only if the J166 *futC* poly-C tract was in-frame (C9) would *lacZ* be in frame. The products were ligated into the EcoRI and XbaI sites 5' of *lacZ* in pUC18 and the ligation reaction was used to transform chemically competent XL-1 Blue cells, inoculated to LB plates containing 100µg/ml carbenicillin, 0.5mM IPTG, and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Sigma), and incubated at 37 °C overnight. Two similarly sized fragments from *galT* (HP0826) from a region without homopolymeric

tracts that differed by one nucleotide were PCR-amplified using primers b(1,4)galTHindIII and b(1,4)EcoRI+240 or +1b(1,4)EcoRI+240 (Table S1). These products were cloned and used as in- or out-of-frame controls. After color development at 4 °C, white and blue colonies, representative of out-of-frame and in-frame products, respectively, were enumerated. Representative white and blue colonies were expanded, plasmid DNA extracted (QIAprep Spin Miniprep Kit, QIAGEN), and sequence analysis performed to examine correlation between phenotype and *futC* genotype.

2.6 Complementation of *futC*

Plasmid pF3::Cm3 was used to disrupt *futC* in strain J166 by inserting a chloramphenicol resistance cassette into the ORF. In brief, J166 cells were transformed with pF3::Cm3, as described [6] and transformants were selected in media containing chloramphenicol (10 µg/mL). Plates were grown for ~5 days, and colonies presumed to represent J166 *futC*/cat isolated and expanded, and insertion of the cassette was examined by PCR using primers futC(−200) fwd and futC(+1050)rev (Table S1). A new suicide vector (pES03) that takes advantage of the requirement of metronidazole to be activated by the oxygen-insensitive NADPH nitroreductase RdxA to inhibit *H. pylori* growth [29] was created by cloning the *rdxA* locus of sequenced strain 26695 into the AatII and NsiI restriction sites in pGEM-T Easy (Promega, Madison, WI). An inverse PCR added a unique NotI site 20bp after the *rdxA* start codon, and deleted the ~200bp that followed. To complement either *futC* disruption or out-of-frame status, in-frame *futC* from MC19 (Table 2) amplified with primers FutC(−140)FNot and FutC(+985)RNotI (Table S1) was cloned into the unique NotI site of pES03, creating pES*futC*_{MC19}. The frame and the orientation of *futC* in the vector were confirmed by sequence analysis.

The metronidazole susceptibility of wild type strain J166, and 98-169 was $0.88 \pm 0.18 \mu\text{g/mL}$ (= sensitive), as measured by E-test (AB BIODISK, Solna, Sweden). pES*futC*_{MC19} was used to transform J166 *futC*/cat and the monkey-recovered derivative 98-169 as described above, using metronidazole (10 µg/mL) selection. The correct insertion of the complementation cassettes within the metronidazole-resistant transformants was examined by PCR (primers rdxA_confirm_fwd and rdxA_confirm_rev, Table S1) (Fig. S2), and subsequently, Le phenotype determined, as described above.

3. Results

3.1 Individual colonies isolated from strain J166 differ from the parental strain in Lewis phenotype

To analyze the phenotypes within an in vitro-passed *H. pylori* population, 379 colonies of strain J166 were isolated, expanded, and prepared for analysis by ELISA using monoclonal antibodies against Le^x or Le^y. The obtained results for both antigens were normalized (see Methods) with the TCMO values for the parental J166 defined as the (1.0, 1.0) point. Two major populations were observed (Fig. 1). Whereas the J166 parental population expresses both Le^x and Le^y, 23% (n=87) of the single colonies expressed an increased level of Le^y (Mean $\pm$ SD TCMO for Le^y= 1.57 ± 0.32), with little or no Le^x expression (0.27 ± 0.27). An inverse phenotype was observed in 274 (72%) isolated colonies (Le^x= 1.26 ± 0.19 , Le^y= 0.39 ± 0.14). The other 18 (5%) colonies had lower levels of both antigens than the parental (Le^x= 0.88 ± 0.23 , Le^y= 0.26 ± 0.20). These results indicate that the parental J166 population is composed of a mixed population of cells with regard to their Lewis phenotype, with two distinct major subpopulations (Fig. 1).

3.2 Individual isolates from strain J166 that vary in Lewis phenotype differ in *futC* genotype

Since *futC* contains a poly-cytosine tract in its ORF, which has been implicated in its phase variation [20,26], we hypothesized that the single colonies with a high Le^x/low Le^y phenotype

predominantly are composed of cells that contain out-of-frame versions of *futC*. Sequence analysis of J166 revealed that an in-frame *futC* contains 9Cs (Fig. S1). Representative J166 single colonies belonging to each of the two different major phenotypic groups observed were selected for further characterization (Table 2). Sequence analysis showed that colony MC6 (low Le^x /high Le^y) contains a C9 poly-C tract, encoding an in-frame *futC* (Fig. 2), whereas MC46 (high Le^x /low Le^y) has a C10 poly-C tract, rendering the gene out-of-frame. Expression of Le^x and Le^y between these two strains was reciprocal, and significantly different ($p < 0.05$). Twelve and 53 nucleotides downstream of the poly-C tract in J166 *futC* are TAA and TGA stop codons in the +1 and -1 frames, respectively (Fig. S1). Sequence analysis performed on other colonies with high Le^x /low Le^y (MC31, MC47, and MC90) or low Le^x /high Le^y (MC19) yielded results similar to those for MC46 and MC6, respectively (Table 2). A poly-A tract (A9) is found directly downstream (15bp) of the poly-C tract in *futC*. In all the strains tested, this homonucleotide tract did not vary in size. For the J166 preparation from the Nashville patient, the *futC* sequence results obtained from the chromatogram (Fig. 2) provide evidence that the cells are mixed with respect to the length of the poly-C tract, consistent with the variety of phenotypes of individual colonies.

3.3 Proportion of J166 cells with *futC* in- or out-of-frame

Based on the evidence that the J166 cell preparation used represented a mixed population with respect to *futC* frame status, we sought to determine the ratio of in-frame/out-of-frame cells within the J166 parental culture. An ~80bp region, including the poly-C tract of *futC* was PCR-amplified from the J166, MC6, and MC46 cell preparations, and the products inserted by directional cloning into pUC18, as described above. As controls, PCR fragments of similar size that did not contain homonucleotide tracts and that were either in-frame or out-of-frame with respect to *lacZ* also were cloned into pUC18. The in-frame and out-of-frame controls each showed the expected results, with low levels (~8%) of background (Fig. 3). For the product from strain MC6, nearly all colonies were blue, consistent with in-frame status, whereas the opposite was found for MC46, as we now expected. The product from strain J166 shows 33.5% in-frame (blue) colonies and 66.5% out-of-frame colonies. In total, these experiments provide further evidence that the J166 cell preparation is mixed with regard to *futC* frame status, and that the single colonies designated MC6 and MC46 are more homogeneous.

3.4 The observed diversity in Le^y phenotype is not due to in vitro passage

Since the J166 parental strain used was relatively close to the original clinical specimen of J166 (<10 passages), we sought to determine whether a single colony of J166 (MC6) would significantly change its Le phenotype during in vitro passage. MC6 cells were grown in vitro, collected for analysis and passed to new media once daily for 25 days (~300 generations). By Le^x and Le^y -specific ELISA there were no consistent changes in either the Le^x or Le^y phenotypes observed (Mean $\pm$ SD TCMO for $Le^x = 0.09 \pm 0.02$; $Le^y = 2.25 \pm 0.30$).

3.5 Complementation of natural Le^y variants with in-frame *futC*

To examine whether the observed *futC* variation is sufficient to explain the change of Lewis phenotype that was present (increase in Le^x , absent Le^y) in monkey-recovered strain 98-169, we sought to restore (in-frame) *futC* in trans, and then determine the subsequent Le phenotypes. A genetic strategy was designed using *futC* complementation plasmid pES*futC*_{MC19}. To test this plasmid, we first disrupted the *futC* ORF in J166 (J166 *futC*/*cat*) by allelic exchange using plasmid pF3::Cm, as described in the Methods section. Lewis phenotype determination by ELISA demonstrated complete loss of Le^y synthesis, as expected (Fig. 4). Subsequently, J166 *futC*/*cat* was transformed with pES*futC*_{MC19} (containing the in-frame *futC* from MC19) and metronidazole-resistant colonies screened by PCR to determine if they had the expected genotype (Fig. S2). Transformant J166 *futC*/*cat*/comp with the correct insertion was shown to

have restored Le^y synthesis and substantial loss of Le^x (Fig. 4). Expression of Le^x and Le^y between these two strains was significantly different ($p < 0.05$).

Strain 98-169, derived from a colony recovered 10 months after challenge of Le^{a+b-} rhesus monkey 85D08, was shown to have originated from input strain J166 [23]. This metronidazole-susceptible (MIC 3.0 ± 0.01 µg/mL) strain was confirmed to have an out-of-frame (C10) *futC* and very low Le^y expression (Fig. 4). Transformation of 98-169 with pES*futC*_{MC19} yielded three metranidazole-resistant colonies, and PCR (Fig. S2) and sequence analysis of both the native and introduced copies of *futC* in each of these three colonies was performed (Fig. S3). For two (colonies 2 and 3), the native *futC* remained out-of-frame, whereas the introduced copy was in-frame, as expected. Colony 1 had two in-frame copies of *futC* but had a similar Le phenotype as the other two 98-169 complemented colonies. For colonies 2 and 3, the *futC* complementation restored Le^y synthesis, accompanied by a substantial loss of Le^x expression (data for colony 3 shown in Fig. 4). Expression of Le^x and Le^y between these two strains was reciprocal, and significantly different ($p < 0.05$). In total, these findings indicate that the observed frameshift in 98-169 *futC* is sufficient to explain the host-dependent Le antigen phenotypic change observed in the experimentally challenged rhesus monkeys [23].

4. Discussion

Our analysis of the Le phenotype of the 379 colonies that were expanded from the J166 preparation that was isolated from a patient revealed that it is composed of a mixture of cells with differing, and essentially reciprocal, Le^x and Le^y phenotypes. Le^x and Le^y expression in representative isolated colonies MC6 and MC46 are significantly different from the parental J166 cell preparation, and also from each other. The ratio observed between the main populations (approximately 25% in-frame, 75% out-of-frame) could either reflect the original ratio from the population of J166 cells within the host, or could represent changes occurring during in vitro passage. Similar proportions were observed after in vitro evolution (~250 generations) of a *lacZ* transcriptional fusion with the phase-variable type III DNA methyltransferase JHP1296 in *H. pylori* strain 1061 [30]. The poly-C tract in JHP1296 contains 14 cytosines, five more than J166 *futC*; slipped-strand mispairing frequency increases with length of the homonucleotide tract [31]. The lack of sustained directional changes in Le^x or Le^y expression during in vitro passage of MC6 indicates that there is no strong in vitro selection for Le phenotype, consistent with our prior findings [19]. These results suggest that the observed diversity in the parental J166 population reflects the original intrahost diversity [19,26], and presumably the gastric distribution [25] of Le phenotypes expressed by the host tissues [21,23].

The two major Le antigen phenotypic populations observed correlated with *futC* frame status. The Le^x and Le^y variation observed within each major population also might be mediated by frame-shifts in the other fucosyltransferases (*futA*, *futB*) [18], growth phase during collection [32], or variation in other genes associated with Le oligosaccharide precursor synthesis [33, 34]. However, the central role of the *futC* genotype in the observed Le^y phenotypes was confirmed by both mutation and complementation analyses. Although the Lewis phenotypes of the predominantly Le^x-expressing strains (J166 *futC/cat* and MC46) and the predominantly Le^y-expressing strains (J166 *futC/cat/comp* and MC6) follow similar trends, they are not identical (Fig. 4). One explanation for the differences may be that populations of MC6 and MC46 accumulated Le variants during their expansion from single colonies. An alternative explanation is translational frame-shifting of *futC*; Wang *et al.* [35] observed that *H. pylori* strain 26695 with *futC* out-of-frame due to slipped-strand mispairing nevertheless may substantially express Le^y. Expression of the 26695 out-of-frame *futC* in an in vitro transcription–translation assay yielded both full-length and truncated proteins. Le^y expression in strain 26695 is ~25 fold higher than in J166-derived strain MC46, suggesting that

translational slippage might not be as efficient in J166, perhaps because J166 *futC* lacks elements critical in 26695 that facilitate frameshifting [35]. A Shine-Dalgarno-like sequence present in the putative translation frameshift cassette located immediately downstream of the poly-C tract is missing, and the predicted stem-loop structure downstream of the Shine-Dalgarno-like sequence has several nucleotide differences [35].

The *futC* poly-A tract immediately downstream of the poly-C tract also might be involved in the observed phase variation [20]. However, long poly-G (or C) sequences are particularly unstable due to higher base-pair dissociation constants [36] and are subject to higher mutation rates than poly-A tracts [37]. Of the 42 homonucleotide tracts associated with phase variation in the AT-rich (61%) *H. pylori* genome, 74% are poly-G or poly-C tracts; this skewed distribution implies selection [9]. Nevertheless, the proximity of the poly-C and poly-A tracts in strain J166 *futC* suggests the possibility of a complex *cis*-acting element involved with Le^y phase variation. Stochastic phenotype-switching mechanisms, as in *futC*, have been assumed to be most favorable when changes in the environment are slow and predictable, and when the environment presents strong selection for particular phenotypes [38]. That slipped-strand mispairing in homonucleotide tracts is overrepresented in *Le* antigen synthesis genes [9] fits this model, as environmental changes (*Le* status of the host) tend to be slow (possibly related to host age) and predictable (only three main *Le* types in hosts).

The alteration of the Lewis phenotype of strain J166 to match that of its host during a 10-month challenge of rhesus monkeys provides evidence for selection of phenotypes [23] governed by the *futC* poly-C tract. The J166 population used in that experiment had *Le* phenotypes of 0.27 ODU for Le^x and 2.02 ODU for Le^y [23], suggesting that in-frame *futC* predominated before challenge. Ten months after the challenge of two Le^{a+b-} phenotype monkeys, only colonies that expressed little or no Le^y were recovered; the out-of-frame *futC* in recovered strain 98-169 and others [23] suggested the locus responsible for the phenotypic shift. Our present complementation analysis in 98-169 with plasmid pES*futC*_{MC19} restoring Le^y phenotype, confirms the sufficiency of *futC* frameshifts to yield the observed host-dependent phenotypic selection. From the three complemented colonies obtained, one had two copies of in-frame *futC* (colony 1, Fig. S3). This new strain had a similar phenotype to the other two 98-169 complemented strains (Fig. S3), and to the J166 complemented *futC* mutant (J166 *futC*/cat/comp). Since this construct differed by only one nucleotide in the *futC* poly-C tract from experimental (out-of-frame) native *futC* strains, it served as an excellent control for our experimental strain, and also allowed us to conclude that harboring two in-frame active copies of the gene does not have a dose effect in J166. Indeed, one copy appears sufficient to modify all (or most) of the LPS molecules in the cell.

In summary, our in vitro analysis of strain J166 revealed the heterogeneity of individual *H. pylori* cell *Le* phenotypes, suggesting that the inoculum used for the monkey challenge already contained similar diversity. This report provides evidence that slipped-strand mispairing is the principal mechanism responsible for the changes in Le^y phenotype in *H. pylori* strain J166 and its variants, for the intrahost phenotypic diversity observed, and for the host-dependent selection for microbial phenotype that occurred during rhesus monkey challenge [23]. Further research will focus on determining the host factors that drive this selection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ELISA	Enzyme Linked Immunosorbent Assay
<i>H. pylori</i>	<i>Helicobacter pylori</i>
Le	Lewis
ODU	optical density units
PBS	phosphate buffered saline
poly-C	poly-cytosine tract
SD	Standard Deviation
TCMO	transformed converted mean ODU

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Fig. 1. Le^x and Le^y expression in single colonies of *H. pylori* strain J166

H. pylori strain J166 was cultured on TSA blood plates, and 379 single colonies were isolated, expanded, and collected for analysis. Lewis expression was determined by ELISA using monoclonal anti- Le^x and anti- Le^y antibodies. Colonies indicated in open diamonds were chosen for sequence analysis. Data are normalized to the Lewis expression of the parental J166 strain. TCMO (transformed converted mean ODU) values are calculated by dividing by the J166 wild type ODU value as a standard (J166 TCMO; $Le^x=1.00$, $Le^y=1.00$, by definition, in open square). Most colonies expressed either high Le^x /low Le^y ($n=290$) or low Le^x /high Le^y ($n=89$). There were few colonies in the low Le^x /low Le^y category (both <1.0 TCMO), except for a clear extension from the high Le^x /low Le^y group. No isolates showed both high Le^x /high Le^y expression, and all expressed either or both Le^x and Le^y .

Fig. 2. DNA sequence analysis for variant single colonies

PCR of the *futC* ORF was performed as described for parental strain J166 and representative colonies MC6 and MC 46, and the DNA sequence of the PCR products determined. Colony MC6 has Le^y >> Le^x expression and contains an in-frame version of *futC*. Colony MC46 has essentially no Le^y and contains an out-of-frame version of *futC*. Cells of the original J166 strain express considerably more Le^x than Le^y. The sequencing results provide evidence that J166 is a mixed population of cells with 9C or 10C *futC* poly-C tracts (red arrow). ODU values (optical density units at 410 nm) of Le expression represent the mean of duplicate determinations.

Fig. 3. Parental strain J166 represents a mixed cell population in relation to *futC* frame PCR fragments containing the poly-C tract of *futC* from strains J166, MC6, and MC46 were inserted by directional cloning into the *lacZ* ORF of pUC18. The primers were designed to express the *lacZ* alpha subunit of pUC18 as a blue colony only if the *futC* poly-C tract was in-frame (with C9). As controls, we studied PCR fragments of similar size that did not contain homonucleotide tracts and that would be expected to produce homogenous populations of colonies based on their pre-determined frame status; a low (~8%) background rate of variation was observed. Total numbers of colonies examined in each cloning category are shown; bars represent the percentage mean $\pm$ SD from 2–3 replicate experiments. Asterisks indicate statistically significant results using Student's T-test, 2-tailed.

Fig. 4. Complementation with in-frame *futC* restores Le^Y expression in strain J166 background Le^X (filled columns) and Le^Y (open columns) expression was measured for *H. pylori* strains J166, MC6, MC46, and 98-169, as well as their mutants or complemented mutants, and data normalized to J166 values. Le^X and Le^Y expression in isolated colonies MC6 and MC46 are significantly ($p < 0.05$) different from parental strain J166 and between each other. Complementation was performed for an *H. pylori* strain in which *futC* was interrupted (J166 *futC/cat*), and also for an Le^Y⁻ variant recovered after monkey challenge (strain 98-169) [23]. In both backgrounds (J166 *futC/cat* and 98-169), in trans introduction of an intact *futC* completely restored Le^Y phenotype and resulted in a reciprocal decrease in Le^X expression, comparable to the differences seen between MC6 (*futC* in-frame) and MC46 (*futC* out-of-frame). Brackets indicate statistically significant ($p < 0.05$) differences for both Le^X and Le^Y expression, using the two-tailed Student's T-test.

Table 1**Bacterial strains and plasmids used in this study**

Strain	Relevant characteristic(s)	Reference or Source
<i>H. pylori</i>		
J166	Wild type strain	[21]
J166 <i>futC</i> / <i>cat</i>	J166 <i>futC</i> :: <i>cat</i>	This study
J166 <i>futC</i> / <i>cat</i> /comp	J166 <i>futC</i> :: <i>cat rdxA</i> :: <i>futC</i>	This study
98-169	J166 isolated after a 10-month challenge of rhesus monkey	[22]
98-169 <i>futC</i> /comp	98-169 <i>rdxA</i> :: <i>futC</i>	This study
MC1-MC379	Single colonies isolated in vitro from culture of parental J166 population	This study
<i>E. coli</i>		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Δ M15 Tn10(Tet ^r)]	Stratagene
Plasmids		
pF3::Cm3	26695 <i>futC</i> interrupted by <i>cat</i> cassette	This study
pES03	26695 <i>rdxA</i> locus interrupted by a NotI site	This study
pES <i>futC</i> _{MC19}	pES03 with in-frame MC19 <i>futC</i> in the NotI site	This study

Table 2
Analysis of isolated J166 colonies with differing Le phenotypes.

Colony designation	Phenotype TCMO (ODU) ^a		<i>futC</i> ^b	
	Le ^x	Le ^y	Number of cytosines in poly-C tract	Frame status
MC6	0.17	1.43	9	In
MC46	1.32	0.10	10	Out
MC19	0.02	1.08	9	In
MC31	0.66	0.08	10	Out
MC47	1.22	0.11	10	Out
MC90	1.11	0.11	10	Out

^aTCMO (transformed converted mean ODU), based on the ratio to reference strain J166, with ODU readings of 2.22 for Le^x, and 0.53 for Le^y, defined as Le^x=1.00, Le^y=1.00.

^bPCR of the *futC* ORF was performed as described (see Methods) for six isolated colonies, and sequence analysis performed. The number of cytosines in the poly-C tract determines *futC* frame status.