Genetic Manipulation of *Helicobacter pylori* Virulence Function by Host Carcinogenic Phenotypes

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**Abstract**

*Helicobacter pylori* is the strongest risk factor for gastric adenocarcinoma, yet only a minority of infected persons ever develop this malignancy. One cancer-linked locus is the cag type 4 secretion system (cagT4SS), which translocates an oncoprotein into host cells. A structural component of the cagT4SS is CagY, which becomes rapidly altered during *in vivo* adaptation in mice and rhesus monkeys, rendering the cagT4SS nonfunctional; however, these models rarely develop gastric cancer. We previously demonstrated that the *H. pylori* cag³ strain 7.13 rapidly induces gastric cancer in Mongolian gerbils. We now use this model, in conjunction with samples from patients with premalignant lesions, to define the effects of a carcinogenic host environment on the virulence phenotype of *H. pylori* to understand how only a subset of infected individuals develop cancer. *H. pylori* cagY sequence differences and cagT4SS function were directly related to the severity of inflammation in human gastric mucosa in either a synchronous or metachronous manner. Serial infections of Mongolian gerbils with *H. pylori* strain 7.13 identified an oscillating pattern of cagT4SS function. The development of dysplasia or cancer selected for attenuated virulence phenotypes, but robust cagT4SS function could be restored upon infection of new hosts. Changes in the genetic composition of cagY mirrored cagT4SS function, although the mechanisms of cagY alterations differed in human isolates (mutations) versus gerbil isolates (addition/deletion of motifs). These results indicate that host carcinogenic phenotypes modify cagT4SS function via altering cagY, allowing the bacteria to persist and induce carcinogenic consequences in the gastric niche.

**Introduction**

Infection with *Helicobacter pylori* is the strongest known risk factor for gastric cancer, a disease that claims >700,000 lives per year (1), yet the precise mechanisms that regulate cancer development in response to this pathogen are less well defined. In many regions of the world, the rates of *H. pylori* infection and gastric cancer are concordant; however, this association is not universal. In Colombia, the prevalence of *H. pylori* is very high throughout the country, but individuals residing in the mountains have high rates of gastric cancer, whereas those on the coast have very low rates (2). Kodaman et al. recently showed that specific interactions between microbial and human genetic ancestries clearly predicted the risk for gastric cancer in Colombia (3). These findings indicate that aberrant coevolution between *H. pylori* and its host may affect pathogenesis.

One specific cancer-linked *H. pylori* locus is the cag pathogenicity island (cagPAI), which encodes a type IV secretion system (T4SS) that translocates the oncoprotein CagA, peptidoglycan, and DNA into host cells (4–6). A structural component of the cagT4SS is CagY, which is required for NF-kB–driven proinflammatory cytokine secretion. CagY encodes for an ~1,900 amino acid protein, which is susceptible to rearrangements, compromising the length and function of the protein. Previous studies have shown that CagY can become rapidly altered during *in vivo* adaptation in mice and Rhesus monkeys, raising the hypothesis that CagY can mediate evasion of the host immune response (7, 8). However, these models rarely develop gastric cancer. In contrast, infection of Mongolian gerbils with *H. pylori* leads to gastric adenocarcinoma in approximately 60% of infected animals (9–12).

To address the hypothesis that a carcinogenic environment within the stomach alters *H. pylori* virulence, we first utilized a unique set of paired *H. pylori* clinical isolates (13). Archival and recent *H. pylori* strain J99 are human clinical strains isolated from a single patient that underwent endoscopy 6 years apart. During this time period, a shift occurred in the pattern of *H. pylori*–induced inflammation in this patient, from antral-predominant to corpus-predominant gastritis, a crucial step in the cascade to gastric carcinogenesis (14). We now use these unique samples, in conjunction with the Mongolian gerbil model of cancer, to define the effects of a carcinogenic host environment on the virulence phenotype of *H. pylori* as a means to understand how only a subset of infected individuals develop gastric cancer in response to this pathogen.
Materials and Methods

Bacterial strains

_H. pylori_ strains were maintained on TSA-blood agar plates. Liquid cultures were prepared in Brucella broth supplemented with 10% newborn calf serum under standard growth conditions. ATCC fully authenticated these cells by short tandem repeat (STR) DNA profiling. After purchase, low-passage vials were frozen and maintained in liquid nitrogen for future use. AGS cells were cocultured with _H. pylori_ at an MOI of 30 for CagA translocation assays or an MOI of 10 for NF-kB assays for all strains included in this study.

Bacterial-epithelial cell cocultures

AGS cells (CRL-1739) were obtained from ATCC and maintained in RPMI-1640 supplemented with 10% fetal bovine serum under standard growth conditions. ATCC fully authenticated these cells by short tandem repeat (STR) DNA profiling. After purchase, low-passage vials were frozen and maintained in liquid nitrogen for future use. AGS cells were cocultured with _H. pylori_ at an MOI of 30 for CagA translocation assays or an MOI of 10 for NF-kB assays for all strains included in this study.

Rodent infections and histopathology

Mongolian gerbils were challenged with _H. pylori_ for up to 16 weeks as previously described (5, 15). Gastric tissues were harvested and gastric injury and quantitative _H. pylori_ cultures were assessed as described (5).

Restriction fragment length polymorphism

PCR products of cagY, cagA, or components of the cagPAI from _H. pylori_ strains were digested with _Ddel_ or _HinfI_ at 37°C and separated in a 6% to 9% polyacrylamide gradient gel. Images from stained gels were acquired using a Biorad ChemiDoc analyzer.

Human samples

Archival and recent _H. pylori_ J99 strains were isolated from gastric biopsy specimens from a single patient 6 years apart (13). Gastric antrum and corpus biopsies from 7 additional patients were obtained via endoscopy. Gastric inflammation and quantitative _H. pylori_ cultures were assessed as described (16).

Statistical analysis

The Mann–Whitney test and one-way ANOVA with a Newman–Keuls posttest were used to compare data groups. Data were plotted and analyzed using Prism (GraphPad Inc).

Results

The _H. pylori_ cancer-linked cagY locus harbors a high level of genetic variability

_cagY_ contains 2 repeat domains that are susceptible to in-frame rearrangements compromising the length of the protein (Supplementary Fig. S2). Sequence analysis of _cagY_ from _H. pylori_ strain 7.13 revealed the presence of 2 different motifs (Motif 2A and 2B). Consensus sequences shown in Supplementary Fig. S3) within repeat region 2 (Supplementary Fig. S2). This particular carcinogenic _H. pylori_ strain has sixteen 2A motifs and six 2B motifs. We therefore used both restriction fragment length polymorphism (RFLP) and DNA sequence changes to identify cagY diversity among isolates used in this study.

Temporal changes in cagY rearrangements and cagT4SS function are directly related to severity of inflammation within an _H. pylori_-colonized human premalignant gastric environment

We first utilized paired _H. pylori_ isolates (strain J99), separated in time by 6 years, that were obtained from the gastric antrum of a single patient. The patient did not receive any antibiotic eradication therapy targeting _H. pylori_ during this time interval. In this patient, total inflammation scores in the gastric antrum were 3-fold higher compared with scores in the corpus at the initial endoscopy (P < 0.05); however, this pattern was reversed 6 years later as the severity of corpus inflammation was approximately 3-fold higher compared with antral inflammation (P < 0.05; ref. 14). At the time of the repeat endoscopy, the gastric pH was 5.0, further indicating that acid secretion was impaired. The transition from antral-predominant to corpus-predominant gastritis suggested that this patient was progressing toward a hypochlorhydric phenotype and may be at a higher risk for intestinal-type gastric adenocarcinoma, although he had not developed this malignancy at the time of the second endoscopy. cagY RFLP profiles differed when the archival J99 isolate was compared with the recent isolate (Fig. 1A). Concordantly, cagY sequence analysis identified 24 single-nucleotide polymorphisms (SNP) when the archival and recent J99 strains were compared. Among the 24 SNPs, 14 encoded synonymous changes and 10 were nonsynonymous (Supplementary Fig. S4). Interestingly, 9 of the 10 nonsynonymous SNPs were located in the cagY repeat domain 2 (Fig. 1B). Comparison of the CagY secondary structures of the archival and recent J99 strains revealed that nonsynonymous SNPs induced changes in the predicted secondary structures (Fig. 1B).

To determine if differences in cagY were related to cagT4SS function, we quantified CagA translocation and NF-kB activation _in vitro_. Archival _H. pylori_ J99, which was isolated from a highly inflamed microenvironment, translocated significantly higher levels of CagA compared with the recent _H. pylori_ J99 isolate. These data mirrored differences in levels of NF-kB activation induced by these strains (Fig. 1C). Thus, cagY sequence differences and cagT4SS function were directly related to the severity of inflammation in a patient who had progressed along a histological cascade toward gastric carcinogenesis.

Output strains from Mongolian gerbils infected with an _H. pylori_ cagY- carcinogenic strain contain different cagY rearrangements, which are linked to changes in cagT4SS function

Based on our results in the human stomach, we next determined the mode and stability of cagY and cagT4SS function in an _H. pylori_ cancer model. Gerbils were infected with the prototype clonal _H. pylori_ cagY carcinogenic strain 7.13 for 12 weeks (Supplementary Fig. S1). Two _H. pylori_ strains recovered from independent gerbils with disparate levels of inflammation and injury were then subjected to cagY RFLP analysis. RFLP profiles revealed variation between one output strain (7.13-2) when compared with the parental strain 7.13 or its sibling output strain 7.13-1 (Fig. 2A). The ability of _H. pylori_ output strain 7.13-2, which induced low inflammation _in vivo_, to translocate CagA or activate NF-kB was significantly attenuated compared with the parental input strain 7.13 or the adapted strain 7.13-1 (Fig. 2B and C). These data indicate that rearrangements in cagY induced by...
prolonged in vivo adaptation in gerbils could modulate function of the cagT4SS.

Mongolian gerbils infected with H. pylori derivative strains harboring distinct cagY rearrangements develop different patterns of disease

To determine if adapted strains with polar in vitro phenotypes could differentially affect pathogenesis in vivo, new populations of gerbils were infected with the H. pylori derivative strains 7.13-1 or 7.13-2 (Supplementary Fig. S1). All challenged animals were successfully colonized, and there were no differences in colonization density between the groups. However, strain 7.13-1, which exhibited considerable potency for CagA translocation and NF-κB activation (Fig. 2B and C), induced high levels of inflammation early (e.g., 8 weeks) post-infection. In contrast, strain 7.13-2 induced a delayed inflammatory response, which began to increase 12 to 16 weeks after infection (Fig. 3A). Preneoplastic lesions were also rapidly induced by strain 7.13-1, and 20% of animals had developed dysplasia 4 weeks after infection, and by 16 weeks after infection, 80% of the 7.13-1–infected animals developed dysplasia and/or adenocarcinoma. In contrast, only 12% of animals infected with strain 7.13-2 developed dysplasia by 16 weeks after infection (Fig. 3B).

Figure 1.
Relationship between cagY SNPs and cagT4SS function in paired human H. pylori isolates. A, cagY RFLP profile of archival and recent H. pylori J99 strains. Arrow, area with different bands. B, Alignment and predicted secondary structure of CagY of archival and recent J99 strains highlighting amino acid substitutions between strains and predicted changes in secondary structure (red boxes). Green, alpha helix; purple, beta-sheets; and red, beta turns. Repeat motifs 2A and 2B are shown in gray. C, NF-κB activation (left axis) and CagA translocation (right axis) in AGS cells following coculture with archival and recent J99 strains. Values are reported as a fold change over uninfected controls (NF-κB) or archival J99 (CagA translocation). Translocation is shown as a ratio of densitometric values. *P < 0.05.
CagA is the ratio of phosphorylated CagA to total CagA densitometric value.

The ability of *H. pylori* to translocate CagA and activate NF-κB in *in vitro* is directly related to the *in vivo* inflammatory phenotype, but inversely related to cancer

Having demonstrated different patterns of injury, readapted output strains from gerbils infected with the *H. pylori* derivative strains 7.13-1 and 7.13-2 were then tested *in vitro* for cagT4SS function. Output isolates from animals infected with the more potent strain 7.13-1 displayed a statistically significant attenuation in the ability to translocate CagA (*P* = 0.008) and activate NF-κB (*P* = 0.004) over time (Fig. 3C and D). In contrast, output strains isolated from animals infected with strain 7.13-2 maintained a relatively stable ability to translocate CagA and activate NF-κB for the duration of infection (Fig. 3C and D). Importantly, there was an inverse relationship between the presence of cancer and cagT4SS function, suggesting that host carcinogenic phenotypes may modify the function of specific *H. pylori* constituents that have been associated with disease (Fig. 3E).

Because *H. pylori* strains 7.13-1 and 7.13-2 exhibited different cagY RFLP patterns, we next determined if the differences observed in CagA translocation and NF-κB activation by the readapted derivatives of *H. pylori* strain 7.13-1 or 7.13-2 were similarly related to new polymorphisms within cagY. Readapted strains isolated from animals infected with *H. pylori* strain 7.13-2 for 16 weeks showed variations in cagY RFLP patterns. Two of the 7.13-2-derived strains (strains 2 and 6) harbored a hybrid RFLP profile containing features present in the profiles of both strains 7.13-1 and 7.13-2; two strains (strains 1 and 3) reverted their RFLP profile to a profile similar to the original parental strain 7.13; and three strains maintained the same profile as input strain 7.13-2 (Fig. 3F). This level of variability mirrored the large variability in inflammatory scores in gerbils infected for 16 weeks with *H. pylori* strain 7.13-2 (Fig. 3A). In contrast to this degree of variability, isolates from animals infected with *H. pylori* strain 7.13-1 showed fewer differences in cagY rearrangements. One 7.13-1-derived strain (strain 4) harbored an intermediate RFLP profile containing elements from strains 7.13-1 and 7.13-2, one (strain 8) had a unique RFLP profile, but six maintained the same profile as the input strain 7.13-1 (Fig. 3F). Interestingly, the readapted strains that reverted their cagY profiles also reverted their ability to translocate CagA and activate NF-κB *in vitro*. In terms of mucosal injury, all *H. pylori* strains that had cagY RFLP profiles similar to strain 7.13-1 induced high levels of inflammation, while strains that harbored cagY RFLP patterns similar to strain 7.13-2 induced low levels of inflammation. Of interest, strains that possessed unique RFLP patterns (e.g., distinct from either strain 7.13-1 or 7.13-2) induced intermediate levels of inflammation (Fig. 3A and F).

Rearrangements in cagY play an essential role in modulating function of the *H. pylori* cagT4SS

Our previous results indicate that the ability of *H. pylori* to induce severe inflammation and gastric injury oscillates in this model of cancer, and this parallels changes in cagT4SS function. To determine the stability of *in vivo*-induced cagY alterations and cagT4SS function, we next selected two output strains from gerbils with the same inflammatory phenotype, but which harbored polar cagT4SS function, and infected new populations of gerbils for 16 weeks (Supplementary Fig. S1). *H. pylori* strain 7.13-1-1 is an *in vivo*-adapted strain obtained from an animal infected for 16 weeks with input strain 7.13-1 and showed an attenuated ability to translocate CagA compared with its parental strain 7.13-1 (Fig. 4A). Strain 7.13-2-7 is a derivative strain from an animal infected for 16 weeks with strain 7.13-2 and exhibited substantial potency in CagA translocation compared with its parental strain 7.13-2 (Fig. 4A). The data shown in Fig. 4B are inflammation scores from new populations of gerbils that were...
infected with either *H. pylori* strain 7.13-1 (parent of 7.13-1-1), strain 7.13-2 (parent of 7.13-2-7), strain 7.13-1-1, or strain 7.13-2-7 for 16 weeks. *In vivo*, the severity of inflammation was directly related to preinoculation cagT4SS function, as determined by CagA translocation, in contrast to strain ancestry. Animals infected with strains 7.13-1 or 7.13-2-7 developed higher injury scores compared with animals infected with strains 7.13-2 or 7.13-1-1 (Fig. 4B and C).

To determine if changes in bacterial phenotypes occurred during these infections, we performed CagA translocation and NF-κB activation assays *in vitro*. Most of the derivative strains from gerbils infected with *H. pylori* strains 7.13-2 or 7.13-1-1 recovered the capacity to translocate CagA. Conversely, only a few strains isolated from animals infected with strains 7.13-1 or 7.13-2-7 lost the ability to translocate CagA (Fig. 4D and E) and these changes in CagA translocation reflected the ability to induce NF-κB (Fig. 4F).

There was a direct correlation between levels of CagA translocation and *cagY* profiles among strains isolated from gerbils infected with *H. pylori* infected with strain 7.13-1-1 (Fig. 4D and G); however, this relationship was not present in strains isolated from gerbils infected with strain 7.13-2-7, as only one isolate (7.13-2-7-6) had a different *cagY* RFLP profile yet maintained the ability to translocate CagA (Fig. 4D and G). Conversely, one strain (7.13-2-7-1) did not express CagA (Fig. 4D), but harbored the same *cagY* RFLP profile of other strains than maintained the ability to express CagA, suggesting a different mechanism of regulation.

In *vivo*–adapted *H. pylori* strains are panmictic populations that harbor different *cagY* rearrangements

We next determined whether reversions of *cagY* genotypes are due to: (i) the presence of panmictic populations prepopulated with isolates harboring different rearrangements of *cagY* or (ii) to...
Figure 4.
de novo rearrangements acquired in vivo. To test this hypothesis, we isolated 12 single colonies from *H. pylori*-adapted strains 7.13-1, 7.13-2, 7.13-1-10, and 7.13-2-16 (Supplementary Fig. S1) and assessed *cagY* RFLP profiles. All single colonies from strain 7.13-1 had identical *cagY* RFLP patterns. Among single colonies from strains 7.13-2, 7.13-1-10, and 7.13-2-16, we identified the presence of clones with different *cagY* rearrangements (Fig. 5A). In total, we found four different RFLP patterns among the collective single colonies. RFLP profiles of strain 7.13-1 single colonies are similar to the original archival strain 7.13; for strain 7.13-2 single colonies, one RFLP profile is similar to 7.13-1 (7.13-2*sc1) and two are different (7.13-2*sc2 and 7.13-2*sc3). For strain 7.13-1-10 single colonies, one RFLP pattern is similar to strain 7.13-1 (7.13-1-10*sc1), and one is different (7.13-1-10*sc2). For strain 7.13-2-16, one RFLP pattern is similar to that of strain 7.13-1 (7.13-2-16*sc1) and one pattern is similar to that of strain 7.13-2-16*sc2, (Fig. 5A).

We next sequenced *cagY* from each of these single colony strains. Compared with the parental H. pylori strain 7.13, sequence analysis identified differences in the number and order of 2A and 2B motifs within the *cagY* repeat region 2 (Fig. 5B). Strain 7.13-1-10*sc1 has a cluster deletion around amino acid 510, which involves the loss of two 2A motifs and two 2B motifs. Strains 7.13-2*sc1, 7.13-2*sc2, and 7.13-2-16*sc2 have an addition of one 2A motif and one 2B motif around amino acid 800. In addition, strain 7.13-2*sc2 also has a deletion that compromises one 2A motif around amino acid 1355. Strain 7.13-1-10*sc2 has an addition of one 2A motif and one 2B motif around amino acid 1220, but this insertion was not detectable by RFLP. Sequences from strains 7.13-1-10*sc1, 7.13-2*sc3, and 7.13-2-16*sc3 did not show any differences when compared with parental strain 7.13 (Fig. 5B). When we compared the predicted secondary structure between single colony strains, the addition or deletion of motifs 2A and/or 2B predicted changes in the length of alpha-helices, as well as additions or deletions of beta sheets (Fig. 5B). Of interest, the mechanism underpinning changes in *cagY* sequences was different in isolates adapted to gerbils (deletion and/or addition of entire motifs) when compared with changes seen in the human *H. pylori* J99 paired strains [SNPs; Fig. 1B].

We also examined cagTASS function among single colony isolates with varying *cagY* profiles. *H. pylori* clones with identical *cagY* RFLP profiles induced similar levels of NF-κB activation; thus clones 7.13-1-10*sc1, 7.13-2*sc3, 7.13-2*sc1, and 7.13-2-16*sc3, which have RFLP patterns similar to the parental strain 7.13, induced the highest levels of NF-κB activation. In contrast, clones 7.13-2*sc1 and 7.13-2-16*sc2, which harbor *cagY* profiles similar to strain 7.13-2, induced the lowest levels of NF-κB activation. Clones 7.13-2*sc2 and 7.13-1-10*sc1 had unique *cagY* rearrangements, with corresponding intermediate and low capacities to activate NF-κB, respectively (Fig. 5A and C). The ability of each of these clones to translocate CagA into AGS cells mirrored their ability to activate NF-κB (Fig. 5C; Supplementary Fig. S5).

To exclude the possibility that changes in the cagPAI exogenous to *cagY* may affect the phenotypes of interest, we performed RFLP analysis of PCR amplicons that spanned the entire cagPAI excluding *cagY*. Global cagPAI RFLP profiles from eight different single colony strains were identical (Supplementary Fig. S6). To further interrogate the entire cagPAI, sequence analysis of the entire cagPAI from two single colony isolates with different CagA translocation phenotypes (7.13-1*sc1 and 7.13-2*sc1) was performed (Supplementary Table S1). The only differences identified were within *cagY* (Supplementary Fig. S7).

To further corroborate the role of *cagY* in modulation of cagPAI function, we genetically exchanged *cagY* among four different strains of *H. pylori* (two with high cagPAI phenotypes, 7.13-1-10*sc1 and 7.13-2*sc3, and two with low cagPAI phenotypes, 7.13-2*sc1 and 7.13-2-16*sc2) using the five different cagY rearrangements found in *H. pylori* single colonies (Fig. 5B; Supplementary Fig. S8). We found that independent of the *H. pylori* recipient background, CagA translocation and NF-κB activation phenotypes were restored or lost based upon the *cagY* phenotype of the donor (Fig. 5D and E; Supplementary Fig. S9). In addition, to define how more refined rearrangements in *cagY* motifs may affect cagPAI function, we exchanged *cagY* between two strains with polar cagPAI phenotypes, 7.13-2-16*sc1 and 7.13-2-16*sc2, but which only differed in a single *cagY* domain 2A (Fig. 5B; Supplementary Fig. S10). We found that *cagY* function could be restored or lost based upon the donor *cagY* motif, implicating this motif in cagPAI function (Fig. 5F).

Mongolian gerbils infected with *H. pylori* single colonies carrying unique *cagY* rearrangements develop differences in the severity of inflammation and disease

To determine whether *H. pylori* single colonies induced differences in disease based on *cagY* RFLP patterns, colonization and inflammation was assessed in gerbils infected with the *H. pylori* single colony isolates. All animals were successfully colonized with the exception of gerbils challenged with the single colony strain 7.13-2*sc1, for which no animals were colonized. Within colonized animals, bacterial density levels were similar (Fig. 6A); however, histopathologic analysis revealed significant differences in inflammation. Gerbils infected with *H. pylori* single colony isolates carrying similar *cagY* rearrangements developed similar levels of inflammation (Fig. 6B). Specifically, gerbils infected with *H. pylori* isolates 7.13-1*sc1, 7.13-2*sc3, 7.13-1-10*sc2, and 7.13-2-16*sc1, which have identical *cagY* RFLP profiles and induce robust CagA translocation (Fig. 5A, 5C), induced high levels of inflammation (Fig. 6B). In contrast, animals infected with isolates 7.13-2*sc2, 7.13-1-10*sc1, and 7.13-2-16*sc2, which harbor different *cagY* profiles and an attenuated ability to translocate CagA (Fig. 5A, 5C), induced low levels of inflammation (Fig. 6B).

Similar to levels of inflammation, only animals infected with strains carrying *cagY* rearrangements linked to robust CagA translocation developed neoplastic lesions (Fig. 6C). In contrast, animals infected with strains carrying *cagY* rearrangements associated with attenuated levels of CagA translocation only developed gastritis (Fig. 6C). These results reinforce the role that *cagY* exerts as a modulator of cagTASS function.

We next analyzed the RFLP profiles of isolated strains. As expected, after 16 weeks, isolated strains harbored differences in *cagY* compared with the input clonal *H. pylori* single colony isolates (Fig. 6D); however, all of these rearrangements represented new RFLP patterns with varying capacities to activate NF-κB and translocate CagA (Fig. 6E and F). Thus, infection with *H. pylori* single colonies harboring a variety of *cagY* RFLP profiles can lead to the generation of new *cagY* rearrangements.

**H. pylori** clinical isolates can harbor synchronous *cagY* rearrangements

We then validated *cagY* diversity observed in our gerbil model by using paired *H. pylori* clinical strains, harvested from 2 different sites (antrum and corpus) within the stomachs of 7 individual
Figure 5.

*H. pylori* gerbil-adapted strains represent panmictic populations harboring diverse *cagY* rearrangements. **A**, Representative *cagY* RFLP profiles of *H. pylori* single colonies generated from strains 7.13-1, 7.13-2, 7.13-1-10, and 7.13-2-16. Arrows, differential bands between RFLP profiles. **B**, Alignment and predicted secondary structure of *H. pylori CagY* parental strain 7.13 and derivative single colony strains. Red boxes highlight gain or loss of motifs 2A and/or 2B compared with parental strain 7.13. Green, alpha helix; purple, beta sheets; and red, beta turns. Repeat motifs 2A and 2B are in gray. **C**, NF-κB activation (black bars) and CagA translocation (white bars) induced by *H. pylori* strains and their corresponding single colony isolates. **D**, NF-κB activation and CagA translocation induced by *H. pylori* strains 7.13-1**sc2** (D) and 7.13-2**sc2** (E) genetically complemented with different *cagY* genes. **F**, NF-κB activation and CagA translocation induced by *H. pylori* strains 7.13-2-16**sc1** and 7.13-2-16**sc2** complemented with either an endogenous *cagY* motif or with a *cagY* motif derived from the sibling strain.
patients during a single endoscopy. For these paired sets of samples, 2 patients (4 and 12) harbored inflammation scores that were higher in the antrum than the corpus, and 5 patients (5, 9, 28, 42, and 44) harbored the reverse (Fig. 7A). In terms of cagY RFLP profiles, strains isolated from patients 4, 5, 28, and 42 harbored different RFLP patterns, while paired isolates from the remaining patients harbored identical profiles (Fig. 7B). Five of the 7 patients harbored either atrophic gastritis or intestinal metaplasia, but there was no significant relationship between site-specific cagY RFLP patterns and premalignant lesions.

We next defined the distribution of cagY rearrangements among single colony isolates (n = 12) harvested from patients 9, 12, 28, 42, and 44. Single colony isolates from the antrum and corpus from patients 9, 12, and 44 displayed no differences in cagY RFLP profiles. In contrast, there were 2 different cagY profiles within antral and corpus single colony isolates from patient 28 and 42. Single colony profiles from patients 28 and 42 were unequally distributed between the antrum and corpus, as the predominant clone in the antrum [patient 28: 8/11 colonies (83%); patient 42: 12/12 colonies (100%)] was the minority clone in the corpus [patient 28: 2/12 colonies (17%); patient 42: 6/12 colonies (50%); Fig. 7C]. Finally, we tested the ability of these isolates to translocate CagA and activate NF-κB in vitro. We found no differences between antrum and corpus isolates from patients 4, 9, 12, and 42. In contrast, there were significant differences in cagT4SS function between antrum and corpus H. pylori isolates from patients 5, 28, and 44 (Fig. 7D). These results in cagT4SS function mirrored NF-κB activation in vitro (Fig. 7E).

Figure 6.
Severity of disease in Mongolian gerbils infected with H. pylori single colony isolates and relationship with cagY rearrangements. Colonization density (A), inflammation scores (B), and disease outcome (C) of gerbils challenged for 16 weeks with H. pylori single colonies carrying specific cagY rearrangements. D–F, cagY RFLP profiles (D), NF-κB activation (E), and phosphorylated and total CagA assays (F) of derivative isolates harvested from gerbils infected for 16 weeks with H. pylori single colony isolates carrying new cagY rearrangements. Statistical differences were calculated by one-way ANOVA using a Newman–Keuls posttest. *, P ≤ 0.05; **, P ≤ 0.01.
Discussion

Our current results indicate that *H. pylori* harbors the capacity to nimbly modify function of its primary virulence constituent during prolonged colonization in a rodent model of gastric carcinogenesis. Chronic infection in gerbils engenders a portfolio of *cagY* isoforms, which are selected in conjunction with the intensity of host inflammatory and carcinogenic phenotypes. Importantly, the development of dysplasia and cancer led to the emergence of attenuated *cagT4SS* phenotypes, but robust function could be restored following adaptation to new hosts. The versatility of *cagY* isoforms and *cagT4SS* function was also validated in both synchronous and metachronous paired human samples and different mechanisms of *cagY* alterations were identified via sequencing. Deletions and additions of entire motifs appeared to predominate in gerbils while the development of single base pair mutations predominated in human samples.

Infection of wild-type mice with *H. pylori cagT4SS*-positive strains frequently leads to deletions within the *cag* island (17, 18), and the mechanism underpinning *cag* dysfunction appears to be in-frame rearrangements in *cagY* (7). In contrast to mice, previous studies have shown that *H. pylori* wild-type *cag* strains colonize gerbils well without loss of *cag* function (19, 20). However, these studies did not examine extensive populations of adapted isolates harvested from a large number of infected gerbils. Our current study has examined changes in *cagY* and the *cagT4SS* in this model in much greater depth through the use of serially passaged strains, investigations of both pools and single colony isolates, and validation in human samples. We now demonstrate that *H. pylori* strains within gerbil stomachs exist as panmictic populations and that *cagT4SS* function can be altered based on the

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**Figure 7.**

Synchronous *cagY* rearrangements in *H. pylori* clinical isolates. **A,** Inflammation scores of human gastric biopsies obtained from the antrum (A) and corpus (C) from the same patient (*n* = 7 patients). **B,** *cagY* RFLP of *H. pylori* isolates from biopsies obtained from the antrum and corpus of the same patient. Boxes indicate differences in RFLP profiles between sibling strains. **C,** Frequency distribution of single colony *cagY* genotypes within isolates obtained from the antrum and corpus from two patients (28 and 42). **D,** CagA translocation and **E,** NF-κB activation of *H. pylori* isolates from the antrum and corpus of seven patients.
severity of disease, which is in contrast to the near-complete ablation of cagT4SS function in mice.

Can long-term serial infections modify gain or loss of cagT4SS function and what is the benefit for H. pylori to harbor mechanisms that can upregulate or downregulate function of this locus? Our current data indicate that the presence of dysplasia or cancer is associated with reduced cagT4SS function. Therefore, we would predict that serial infections would not enhance CagA translocation and/or the ability to activate NF-κB if there is development of these lesions. However, this needs to be tested formally in the future. In terms of serially infecting derivative strains with minimal cagT4SS function, our data in Fig. 4 have shown that strain 7.13-1-1, a derivative strain with low cagT4SS function, when reinfected into gerbils, induces variability in inflammation scores, CagA translocation indices, and NF-κB activation levels (Fig. 4B, D, E, and F). Based on our results investigating single colony isolates (Fig. 6), we posit that this reflects a panmictic pool of clones with variable cagY function within preinoculation strain 7.13-1-1. In terms of strains that never acquire the ability to translocate CagA or activate NF-κB, our data in Fig. 5 demonstrate that two derivative strains, 7.13-2^w and 7.13-2-16^w, have little if any ability to translocate CagA or activate NF-κB, and their cagY RFLP profiles are identical (Fig. 5A). Therefore, we plan in future experiments to utilize these strains in long-term adaptation studies to more carefully discern whether chronic and repeated serial infections may lead to a gain of cagT4SS function. One downstream ramification of CagA translocation is acquisition of iron sources that fuel H. pylori replication (21). Under conditions of iron deprivation, therefore, it may be beneficial for H. pylori to augment function of the cagT4SS and deliver higher payloads of CagA into host epithelial cells. H. pylori also harbors multiple pathogen-associated molecular patterns (PAMP) that interact differently with innate immune receptors than the respective counterparts in other mucosal pathogens. H. pylori FlaA is a noninflammatory molecule in terms of its ability to activate TLR5 (22). H. pylori LPS contains an anergic lipid A core that induces an attenuated TLR4-mediated response (23, 24). We and others have shown that deacetylation of peptidoglycan allows H. pylori to evade host clearance (5, 23–27). Thus, H. pylori has evolved to express an array of diverse phenotypes to subvert obstacles presented by the host (28), and the ability of H. pylori to alter cagY genotypes is yet another mechanism within the repertoire of this organism to evade host defenses.

In conclusion, we have utilized a robust rodent model of gastric cancer that closely recapitulates human disease to define alterations in H. pylori pathogenesis in vivo. Our findings demonstrate that cagT4SS function is typically maintained during prolonged colonization in this model but that the intensity of cagT4SS function can vary and is related to host disease phenotypes. cagY genotypes are altered concordantly with cagT4SS function and can similarly vary in paired human samples obtained from different regions of the stomach or separated in time. The use of this model will facilitate more detailed investigations in the future that can identify new targets and therapeutic strategies to reduce the risk of cancer associated with this pathogen.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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