CagY-dependent regulation of type IV secretion in *Helicobacter pylori* is associated with alterations in integrin binding

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ABSTRACT

Strains of *Helicobacter pylori* that cause ulcer or gastric cancer typically express a type IV secretion system (T4SS) encoded by the *cag* pathogenicity island (PAI). CagY is an ortholog of VirB10 that, unlike other VirB10 orthologs, has a large middle repeat region (MRR) with extensive repetitive sequence motifs, which undergo CD4+ T cell-dependent recombination during infection of mice. Recombination in the CagY MRR reduces T4SS function, diminishes the host inflammatory response, and enables the bacteria to colonize at a higher density. Since CagY is known to bind human $\alpha_5\beta_1$ integrin, we tested the hypothesis that recombination in the CagY MRR regulates T4SS function by modulating binding to $\alpha_5\beta_1$ integrin. Using a cell-free microfluidic assay, we found that *H. pylori* binding to $\alpha_5\beta_1$ integrin under shear flow is dependent on the CagY MRR, but independent of the presence of the T4SS pili, which are only formed when *H. pylori* is in contact with host cells. Similarly, expression of CagY in the absence of other T4SS genes was necessary and sufficient for whole bacterial cell binding to $\alpha_5\beta_1$ integrin. Bacteria with variant *cagY* alleles that reduced T4SS function showed comparable reduction in binding to $\alpha_5\beta_1$ integrin, though CagY was still expressed on the bacterial surface. We speculate that *cagY*-dependent modulation of *H. pylori* T4SS function is mediated by alterations in binding to $\alpha_5\beta_1$ integrin, which in turn regulates the host inflammatory response so as to maximize persistent infection.

IMPORTANCE

Infection with *H. pylori* can cause peptic ulcers, and is the most important risk factor for gastric cancer, the third most common cause of cancer death worldwide. The major *H. pylori* virulence factor that determines whether infection causes disease or asymptomatic colonization is the type IV secretion system (T4SS), a sort of molecular syringe that injects bacterial products into gastric epithelial cells and alters host cell physiology. We previously showed that recombination in CagY, an essential T4SS component, modulates the function of the T4SS. Here we found that these recombination events produce parallel changes in specific binding to $\alpha_5\beta_1$ integrin, a host cell receptor that is essential for T4SS-dependent translocation of bacterial effectors. We propose that CagY-dependent binding to $\alpha_5\beta_1$ integrin acts like a molecular rheostat that alters T4SS function and modulates the host immune response to promote persistent infection.

INTRODUCTION

Helicobacter pylori infection most often causes only asymptomatic gastritis, but is considered an important human pathogen because it is the major risk factor for development of peptic ulcer disease and gastric adenocarcinoma (1), the third most common cause of cancer death. On the other hand, *H. pylori* infection may also have beneficial effects, particularly prevention of chronic diseases that have increased in frequency in developed countries as the prevalence of *H. pylori* has declined (2). The bacterial virulence factor most strongly associated with the outcome of *H. pylori* infection is the *cag* pathogenicity island (*cag*PAI), a ~40 kb DNA segment that encodes a type IV secretion system (T4SS). When *H. pylori* comes in contact with the gastric epithelium, it assembles the T4SS pilus (3), through which it injects the CagA oncoprotein into host cells (4). Other T4SS-dependent effectors have also been identified, including DNA (5), peptidoglycan (6) and heptose-1,7-bisphosphate, a metabolic precursor in lipopolysaccharide biosynthesis (7-9). Together, T4SS injection of effector molecules results in complex changes in host cell physiology that include cytoskeletal rearrangements, disruption of tight junctions, loss in cell polarity, and production of interleukin 8 (IL-8) and other proinflammatory cytokines (4, 10).

Host cell expression of β1 integrins is required for T4SS-dependent translocation of CagA (11, 12), and presumably other effectors as well. Four *cag*PAI proteins essential for T4SS function have been found to bind β_1 integrins, though the details are unclear and some reports are contradictory. The first to be described was CagL, an RGD-dependent ligand for $\alpha_5\beta_1$ integrin that presumably mimics fibronectin, an intrinsic host integrin ligand (11). An RGD helper motif in CagL (FEANE) may also be important (13). However, other studies have failed to demonstrate CagL binding to β_1 integrins (12), have yielded discrepant results about the role of CagL polymorphisms (14-16), or have identified completely different integrin binding partners, including $\alpha_V\beta_6$ and $\alpha_V\beta_8$ (17). CagA, CagI and CagY have also been shown to bind β_1 integrin using yeast two hybrid, immunoprecipitation, and flow cytometry approaches (12). However, *H.*

pylori binding to integrins has only occasionally been performed with intact bacterial cells (12, 18), and the role of the *cag*PAI-encoded proteins for integrin binding has not yet been examined in the context of a fully assembled T4SS.

It has long been known that passage of *H. pylori* in mice results in loss of T4SS function (19, 20). We previously demonstrated that this is typically a result of recombination events in *cagY* (21), a *virB10* ortholog that contains in its middle repeat region (MRR) an extraordinary series of direct DNA repeats that are predicted to encode in-frame insertions or deletions in a surface-exposed region of the protein (22). Recombination events in the *cagY* MRR lead to expression of an alternative CagY allele that can modulate T4SS function, including induction of IL-8 and translocation of CagA (21). This modulation can occur in a graded fashion, and cause both gain and loss of T4SS function (21). More recently, we demonstrated that IFNγ and CD4+ T cells are essential for *cagY*-mediated loss of T4SS function, which can rescue colonization in *IL10-/-* mice that have an exaggerated inflammatory response to *H. pylori* infection (23). Together, these results suggest that *cagY* recombination serves as an immune sensitive molecular rheostat that “tunes” the host inflammatory response so as to maintain persistent infection.

Here we examined the mechanism by which recombination in *cagY* alters T4SS function. Since CagY forms the spokes of a T4SS core complex, together with CagX, CagM, CagT and Cag3 (24, 25), one possibility is that changes in the MRR alter T4SS function by modifying essential protein-protein interactions, or changing the pore through which effectors must travel. Alternatively, since CagY recombination occurs in the MRR, which is predicted to extend extracellularly, allelic variation in CagY might alter integrin binding. At first glance this seemed unlikely since there are multiple *cag*PAI proteins that bind integrins. Surprisingly, our results demonstrate that indeed recombination in the CagY MRR alters binding to β_1 integrin, which in turn modulates T4SS function. Moreover, the CagY MRR is expressed on the bacterial surface even in the absence of a T4SS pilus. We propose that CagY is a bifunctional protein

114 that contains a VirB10 domain that is an essential part of a complete T4SS structure, and an

115 MRR region that mediates close contact to the host cell and modulates T4SS function.

RESULTS

H. pylori binds to α5β1 integrin in a host cell-free assay

Previous studies analyzed binding of *H. pylori* to β_1 integrins by protein-protein interaction assays, protein to host cell binding, or bacterial co-localization to β_1 integrin on host cells *in vitro* (11, 12). To demonstrate binding of intact live *H. pylori* to β_1 integrin, we developed a microfluidic assay in which human recombinant $\alpha_5\beta_1$ integrin was coated on glass cover slips, which served as the substrate of a flow channel (Fig 1A). Fluorescently stained bacteria were flowed through the channel at a defined shear stress (~1 dyne/cm^2), microscopic images were recorded, and immobilized fluorescent bacteria were counted. To validate the microfluidic assay, we first analyzed binding of *Escherichia coli* expressing *Yersinia* invasin, a well-characterized β_1 integrin ligand (26). *Yersinia* InvA was expressed in *E. coli* after IPTG stimulation (Fig S1A) and was presented on the bacterial cell surface (Fig 1B). *E. coli* harboring the plasmid vector alone served as a negative control. *E. coli* expressing InvA and fluorescently stained with either DiO or DiD showed markedly increased binding to $\alpha_5\beta_1$ compared to control *E. coli* with vector alone (Fig 1C). Similar results were obtained when InvA and control strains were mixed 1:1 and analyzed simultaneously, which permitted direct comparison in the same flow channel, and limited variability that might otherwise arise from differences in integrin density or flow disturbances on glass coverslips (Fig S1B).

Fluorescently stained *H. pylori* was also readily visualized adherent to $\alpha_5\beta_1$ integrin (Fig 1D). *H. pylori* strains J166 and PMSS1 both attached to $\alpha_5\beta_1$ integrin in a concentration-dependent manner and reached saturation at an optical density (OD_{600}) of 0.8 (Fig 2A). This correlates with approximately 4x10^8 bacterial cells per ml and was used for all subsequent experiments. Binding was blocked by pre-incubating the integrin-coated cover slips with P5D2 anti-β_1 antibody, which sterically inhibits integrin-dependent binding (Fig 2B). Allosterically stabilizing the $\alpha_5\beta_1$ integrin in the low affinity conformation by pre-incubation with SG19 antibody decreased *H. pylori*-integrin binding, while the TS2/16 antibody that locks $\alpha_5\beta_1$ in the high affinity

conformation yielded binding similar to that of an isotype control, indicating that the majority of derivatized $\alpha_5\beta_1$ is active (Fig 2B). This was also supported by the observation that pretreatment of the microfluidic channels with Mn^{2+} to lock $\alpha_5\beta_1$ integrin in the high affinity state yielded *H. pylori* adherence similar to that with the TS2/16 antibody and the isotype control (Fig 2B). Adherence to $\alpha_5\beta_1$ integrin was greater than to $\alpha_4\beta_1$ and $\alpha_L\beta_2$ (Fig 2C). Thus, live whole cell *H. pylori* binds specifically and in a conformation dependent manner to $\alpha_5\beta_1$ integrin.

H. pylori adherence to $\alpha_5\beta_1$ integrin in a host cell-free assay is dependent on CagY

To determine if the *cag*PAI, or any of the putative integrin binding partners (CagA, CagI, CagL or CagY), are responsible for $\alpha_5\beta_1$ integrin binding of intact *H. pylori*, we compared deletion mutants of *H. pylori* J166 to the wild type (WT) control. The number of adherent mutant and WT *H. pylori* per field of view was determined, and the results were analyzed as the percent adherence of the mutant compared to WT. Initial control experiments demonstrated that WT and selected mutant strains stained with similar efficiency with both dyes (Fig S2A), and that adhesion was independent of the dye and was similar whether strains were analyzed individually or competitively (Fig S2B). Adherence to $\alpha_5\beta_1$ integrin was markedly reduced by deletion of the entire *cag*PAI (Δ*cag*PAI), but not by deletion of *cagE* (Δ*cagE*) or *cagI/L* (Δ *cagI/L*) (Fig 2D). Deletion of *cagA* (Δ*cagA*) produced a small reduction in adherence to $\alpha_5\beta_1$ integrin but the difference was not statistically significant (P=0.25). In contrast, integrin adherence by the *cagY* deletion mutant (Δ*cagY,* shown schematically in Fig 3) was significantly reduced to a level similar to Δ*cag*PAI (Fig 2D). Blocking by treatment with anti-β_1 antibody demonstrated β_1-specific binding in Δ*cagA, *Δ*cagI/L, and* Δ*cagE* mutants (Fig 2E), which all produced CagY as demonstrated by immunoblot (Fig S3A). Δ*cagY* showed only residual adherence that was not β_1-specific (Fig 2E). Together, these results demonstrate that in this host cell-free system, adhesion of *H. pylori* to $\alpha_5\beta_1$ integrin under physiological levels of shear stress is mediated predominantly by CagY.

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169 **CagY-mediated integrin binding is independent of the T4SS pilus**

170 *H. pylori* T4SS pilus formation is thought to require host cell contact (27), though this has
171 never been formally demonstrated. Since *H. pylori* attachment to integrin in the flow channel
172 occurs in the absence of host cells, this suggests that *H. pylori* can bind to $\alpha_5\beta_1$ integrin
173 independent of the T4SS pilus. To examine this further, we used field emission scanning
174 electron microscopy (FEG-SEM) to image the T4SS pili in *H. pylori* WT and Δ*cag*PAI, co-
175 cultured with or without AGS gastric epithelial cells. Numerous pili were observed on WT *H.*
176 *pylori* J166, but only in the presence of AGS cells (Fig 4). As expected, no pili were detected on
177 J166Δ*cag*PAI. The same results were found for *H. pylori* PMSS1 WT and Δ*cag*PAI (Fig S4).
178 Culture of *H. pylori* together with $\alpha_5\beta_1$ integrin also failed to induce pilus formation (data not
179 shown). Therefore, under shear flow in this cell-free system, CagY-mediated binding to $\alpha_5\beta_1$
180 integrin does not require formation of the T4SS pilus. To further demonstrate that CagY is
181 sufficient for integrin binding in the absence of the T4SS pilus, all of the PAI genes were deleted
182 except *cagX* and *cagY*, which are transcribed as an operon from a putative promoter located in
183 *cag9*, upstream of *cagX* (28, 29). This mutant, designated *cagXY*, is shown schematically in Fig
184 3 compared to J166 WT and Δ*cagY.* J166 *cagXY* expresses CagY on the bacterial surface (Fig
185 5A,C) but fails to induce a robust IL-8 response in AGS cells due to the lack of a T4SS (Fig S5).
186 In the flow channel $\alpha_5\beta_1$ integrin binding assay, J166 *cagXY* binds at a level similar to J166 WT
187 (Fig 5E). To exclude a role for CagX, we deleted all *cag*PAI genes and stitched *cagY* directly to
188 the promoter in *cag9*, creating J166 *cagY*. Similar to J166 *cagXY*, J166 *cagY* fails to induce IL-8
189 (Fig S5), but expresses CagY and binds to $\alpha_5\beta_1$ integrin similarly to J166 WT (Fig 5B,D and F).
190 Together these results suggest that in this assay, *H. pylori* binds to $\alpha_5\beta_1$ integrin predominantly
191 via a CagY-dependent mechanism, but independently of T4SS pilus formation. This conclusion
192 is also supported by the observation that integrin binding in J166 Δ*cagI/L* and Δ*cagE,* which do
193 not form a T4SS pilus (27), is similar to WT (Fig 2D).

The CagY MRR is necessary for integrin binding

The topography of CagY in the bacterial cell is poorly understood. Proteomic studies suggest that it may be located in the cytoplasmic membrane, or perhaps span the inner and outer membranes [18], similar to what has been demonstrated for the *Escherichia coli* VirB10 (30). However, CagY is much larger than other VirB10 orthologs, and includes two membrane spanning domains that flank the MRR, which previous studies suggested may be localized to the bacterial surface (31). Surface localization is also apparent in deletion mutants of *cagA*, *cagI/L* and *cagE* (Fig S3B), and in J166 *cagXY* (Fig 5A and S3C) and J166 *cagY* (Fig 5D), which do not make a T4SS pilus. We next constructed an unmarked in-frame deletion of the MRR (designated J166Δ$cagY_{MRR}$), which is shown schematically in Fig 3. J166Δ$cagY_{MRR}$ does not induce IL-8 (Fig S5) or bind $\alpha_5\beta_1$ integrin in the flow channel (Fig 5E), and, as expected, shows no surface localization of CagY using antibody directed to the MRR (Fig 5C). However, J166Δ$cagY_{MRR}$ has an in-frame deletion and produces CagY that can be detected with antibody to the VirB10 portion of CagY (Fig 5A). Together, these results suggest that the *H. pylori* CagY MRR is expressed on the bacterial surface, is required for the binding of $\alpha_5\beta_1$ integrin in a T4SS-independent manner, and is essential for T4SS function.

Variation in the motif structure of the CagY MRR alters binding to $\alpha_5\beta_1$ integrin and T4SS function.

We previously demonstrated, using mouse and non-human primate models, that recombination in the *cagY* MRR regulates T4SS function (21, 23), though the mechanism is unknown. Since we have now shown that the MRR is also required to bind $\alpha_5\beta_1$ integrin in the flow channel, we hypothesized that recombination in *cagY* modulates T4SS function by altering the efficiency of *H. pylori* adhesion to $\alpha_5\beta_1$ integrin. To test this hypothesis, we compared IL-8 induction to integrin adhesion, using three groups of *H. pylori* strains, each with several isogenic

variants bearing unique *cagY* alleles that were previously documented to confer changes in IL-8 induction. First, we examined four isogenic *H. pylori* J166 strains bearing different *cagY* alleles, which arose naturally during infection of mice and were transformed into the WT parent strain (21). All four strains express an unmarked CagY that differs only in the motif structure of the MRR (Fig 6A). Two of the strains induce IL-8 and translocate CagA similarly to WT J166, and two have a non-functional T4SS (21). Consistent with our hypothesis, changes in the J166 CagY MRR that reduced IL-8 also showed a marked and commensurate reduction in adhesion to $\alpha_5\beta_1$ integrin (Fig 6B). Parallel experiments with isogenic strains of *H. pylori* PMSS1 bearing a unique CagY MRR that altered T4SS function (23) showed similar results (Figs 6C and 6D). Finally, we examined the relationship between induction of IL-8 and integrin binding in paired clonal *H. pylori* isolates recovered from a human patient over a period of 7.4 years (KUS13A and KUS13B), and which differed in the CagY MRR and in T4SS function (23). Again we found that MRR-dependent adhesion of each *H. pylori* isolate to $\alpha_5\beta_1$ integrin was in most cases commensurate with the level of IL-8 induction (Fig 6E and 6F). Together these results suggest that recombination in *cagY* modulates T4SS function by altering *H. pylori* attachment to $\alpha_5\beta_1$ integrin.

Variant CagY amino acid motifs that differ in integrin binding and T4SS function are expressed on the bacterial surface

Recombination of *cagY* could modulate integrin binding by changing its amino acid motif structure, but it might also change its level of expression or surface localization. Although in some strains the level of CagY expression appears decreased (e.g. Fig 6A, strain 3), this likely reflects a marked reduction in size of the MRR and reduced antibody recognition. We detected no relationship between MRR expression on western blot and either *H. pylori* adhesion to integrin or induction of IL-8 (Fig 6). CagY MRR was expressed on the bacterial surface in isogenic *H. pylori* PMSS1 strains that differed only in their MRR, and also showed no

246 relationship to T4SS function or integrin binding (Fig 7A). Analysis of fluorescence intensity
247 normalized to DAPI staining demonstrated quantitatively that CagY was expressed on the
248 bacterial surface at similar levels, with no detection in the negative control (Fig 7B). Quantitation
249 of expression on the bacterial surface of isogenic *cag*PAI mutants of J166 similarly showed no
250 relationship to T4SS function or integrin binding, though all MRR variants showed reduced
251 expression (Fig S3D), perhaps related to the reduction in number of MRR motifs. These results
252 suggest that changes in the motif structure of CagY on the bacterial surface modulate T4SS
253 function by altering bacterial adhesion to $\alpha_5\beta_1$ integrin, rather than altering surface presentation
254 of CagY.

DISCUSSION

H. pylori persistence in the gastric mucosa is often attributed to evasion of the innate and adaptive immune response, including antimicrobial peptides (32), toll like receptor signaling (33, 34), and T cell proliferation (35, 36), as well as promotion of a regulatory T cell response (37). However, the very presence in most strains of the *cag*PAI, which promotes the host immune response (38, 39), and the uniform occurrence of gastritis in infected patients, suggest the possibility that the host inflammatory response may at the same time actually promote *H. pylori* colonization, a concept that has recently been elegantly demonstrated for several enteric pathogens (40). This is supported by observations of functional antagonism between some *H. pylori* virulence factors such as the CagA oncoprotein and the VacA cytotoxin (41, 42), and by recent evidence that CagA-dependent inflammation may be important for acquisition of essential nutrients such as iron (43, 44) and zinc (45). This more nuanced view of the relationship between *H. pylori* and the host immune response suggests that the overarching strategy used by *H. pylori* to persist in the stomach might be better characterized as immune regulation rather than simply immune evasion.

CagY is an essential component of the *H. pylori* T4SS that may be well-suited to serve this immune regulatory function. The *cagY* gene has in its middle repeat region (MRR) a series of direct DNA repeats that *in silico* predict in-frame recombination events. Recombination in the *cagY* MRR is in fact common, since variants can be readily detected *in vitro*, though it remains possible that the frequency is increased in response to unknown host signals. We previously showed that *cagY* recombination *in vivo* yields a library of insertions and deletions in the MRR, which maintain CagY expression but frequently alter T4SS function (21). CagY-dependent modulation of T4SS function is graded—more like a rheostat than a switch—and can yield variants that confer both gain and loss of function in vivo (21, 23). Adoptive transfer and knockout mouse experiments demonstrate that development of variant *cagY* alleles requires a CD4+ T cell- and IFNγ-dependent immune response (23). Thus, *cagY* recombination can

281 modulate T4SS function and may be a bacterial strategy to both up- and down- regulate the
282 host immune response to promote persistent infection.

283 Here we addressed the mechanism by which recombination in the MRR alters T4SS
284 function. Since CagY is a ligand for $\alpha_5\beta_1$ integrin, which is essential for T4SS function, we
285 hypothesized that changes in the amino acid motif structure from recombination in the MRR
286 might alter integrin binding and modulate T4SS function. Analysis of whole bacterial cells in a
287 microfluidic assay demonstrated CagY-dependent and integrin conformation-specific binding to
288 $\alpha_5\beta_1$, which correlated closely with T4SS function in isogenic variants that differed only in the
289 MRR region of CagY. This binding was independent of the T4SS pilus, which was not formed
290 under these cell-free conditions, though the MRR was expressed on the bacterial surface as
291 described previously (31). Moreover, we could detect the MRR on the bacterial surface even
292 when the entire *cag*PAI was deleted except for *cagY* and the upstream promoter. Binding to
293 $\alpha_5\beta_1$ integrin was not dependent upon CagA, CagE, or CagL, which was originally identified as
294 the ligand for $\alpha_5\beta_1$ integrin (11). While CagL is clearly essential for T4SS function, more recent
295 studies have suggested that it binds $\alpha_V\beta_6$ and $\alpha_V\beta_8$ integrin and not $\alpha_5\beta_1$ (17). The results from
296 yeast two-hybrid studies (12) also identified CagI as a β_1 integrin binding partner, which we
297 could not confirm in whole bacterial cells.

298 Previous studies have found that the VirB10 ortholog at the C-terminus of CagY bound
299 to $\alpha_5\beta_1$ integrin, but not the MRR region. However, these studies examined protein-protein
300 interactions by yeast two-hybrid and immunoprecipitation or by surface plasmon resonance (12,
301 46), which may not reflect binding in a whole bacterial cell. Since the isogenic *cagY* variants
302 examined here differed only in the MRR, and deletion of the MRR eliminated $\alpha_5\beta_1$ integrin
303 binding, our results suggest that the *H. pylori* MRR is required for binding to $\alpha_5\beta_1$ integrin in an
304 intact bacterial cell. However, we have not directly examined MRR binding to $\alpha_5\beta_1$ integrin, so
305 the MRR may not itself be an integrin ligand, but instead may modulate binding of the VirB10
306 domain of CagY. We have been unable to demonstrate CagY-dependent adherence to $\alpha_5\beta_1$

integrin on AGS gastric epithelial cells in our microfluidic assay (data not shown), which may reflect the multiple binding partners, including *cag*PAI components, as well as HopQ, BabA, SabA, and other outer membrane adhesins (47, 48). Others have also found no difference in binding to AGS cells between WT and Δ*cag*PAI (12).

The topology of CagY in the bacterial membrane, and the accessibility to the $\alpha_5\beta_1$ integrin, also remain areas of uncertainty. Integrins are generally found in the basolateral compartment, which would not normally be accessible to *H. pylori* on the apical cell surface. However, *H. pylori* binds preferentially at tight junctions in cell culture and in gastric tissue, leading to disruption of the integrity of the epithelial layer (49). Moreover, recent studies suggest that *H. pylori* HtrA, an essential serine protease, cleaves occludin, claudin-8, and E-cadherin, which opens cell-cell junctions and may explain how *H. pylori* could bind integrins *in vivo* (50-52). *H. pylori* binding to CEACAMs (53, 54) or other yet identified cell receptors may also induce redistribution of integrins from the basolateral to the apical cell surface, making them accessible to CagY. It also remains unclear precisely how CagY is localized in the bacterial cell membrane. Elegant cryo-electron microscopy studies have demonstrated that the VirB10 orthologue in the *Escherichia coli* plasmid conjugation T4SS forms part of a core complex that spans the inner and outer bacterial membranes (30). However, the topology in *H. pylori* appears different, as recent electron microscopy studies suggest that the core complex is much larger than that in *E. coli*, and is composed of 5 proteins (rather than 3), including CagX, CagY, CagM, CagT, and Cag3 (25).

In conclusion, these studies demonstrate that CagY modulates attachment to $\alpha_5\beta_1$ integrin independently of the T4SS pilus in a manner that depends on the MRR motif structure. It is tempting to speculate that CagY-mediated alteration in integrin binding is also mechanistically linked to T4SS function, since they are strongly correlated (Figure 6). For example, surface expression of an integrin-binding motif may promote intimate epithelial cell contact, which in turn serves as a nucleation signal to promote expression of the T4SS pilus,

333 further enhancing integrin binding and injection of effector molecules. Such a scenario might
334 entail MRR-dependent integrin signaling, including activation of focal adhesion kinase (FAK)
335 and the Src family kinase, though others have shown that only the extracellular domains of the
336 β_1 integrin are important for CagA translocation (12). On the other hand, it is logically possible
337 that changes in the MRR affect integrin binding and T4SS function independently. Though the
338 details remain to be elucidated, we hypothesize that CagY-dependent binding to $\alpha_5\beta_1$ integrin
339 serves as a molecular rheostat that "tunes" the optimal balance between the competing
340 pressures of gastric inflammation, which serves a metabolic function for the bacterium on the
341 one hand, but comes at a cost of exposure to immune pressure, decreased bacterial load, and
342 decreased possibility of transmission to a new host.

MATERIALS AND METHODS

Construction and culture of *E. coli* expressing InvA

Plasmid pRI253 (kindly provided by Ralph Isberg, Tufts University, Boston, MA) contains the *invA* gene from *Yersinia pseudotuberculosis* under the control of a phage T7 RNA polymerase promoter (55). To create a negative control, the *invA* gene was cut out from the plasmid using restriction enzymes EcoRI and HindIII. The recircularized plasmid pRI253Δ*invA* and the original plasmid pRI253 were transformed into competent *E. coli* BL21 (Invitrogen) according to the manufacturers' instructions. *E. coli* strains were cultured overnight at 37°C in Luria Bertani (LB) broth supplemented with 5 mg/liter carbenicillin. Overnight cultures were diluted to an OD_{600} of 0.05, cultured for an additional 2-3 h, followed by addition of 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and another 2 h of incubation to induce InvA expression.

H. pylori strains and culture conditions

Wild type *H. pylori* strains were cultured on Brucella agar or in Brucella broth (BBL/Becton Dickinson, Sparks, MD) supplemented with 5% heat-inactivated newborn calf serum (Invitrogen, Carlsbad, CA) and antibiotics (trimethoprim, 5 mg/liter; vancomycin, 10 mg/liter; polymyxin B, 2.5 IU/liter, amphotericin B, 2.5 mg/liter). *H. pylori* mutant strains were cultured as for wild type, but with the addition of kanamycin (25 mg/liter), chloramphenicol (5 mg/liter), or streptomycin (10 mg/liter) as appropriate (all antibiotics from Sigma). *H. pylori* liquid cultures were grown overnight to an optical density at 600 nm (OD_{600}) of approximately 0.3 to 0.4. All *H. pylori* cultures were grown at 37°C under microaerophilic conditions generated by a fixed 5% O_2 concentration (Anoxomat, Advanced Instruments, Norwood, MA). A complete list of strains is shown in Table 1.

368

369 **Construction of *H. pylori* mutants**

370 Six mutants in *H. pylori* J166 were constructed (Table 1). For J166Δ*cagA*, J166Δ*cagI/L*
371 and J166Δ*cagE,* DNA fragments upstream and downstream of the respective gene deletion
372 were PCR amplified using primers (Table S1) with restriction sites that permitted ligation to a
373 kanamycin resistance gene (*aphA*), and insertion into the multiple cloning site of pBluescript
374 (Stratagene, La Jolla, CA). The resulting plasmid was transformed into *E. coli* TOP10
375 (Invitrogen) according to the manufacturers' instructions, and transformants were grown
376 overnight on Luria-Bertani (LB) plates containing kanamycin. Resistant colonies were inoculated
377 in selective LB broth and plasmids from the resulting culture were purified with a QIAprep® Spin
378 Miniprep kit (Qiagen). Plasmids were sequenced and digested with appropriate enzymes for
379 verification of correct construction prior to natural transformation of *H. pylori* with kanamycin
380 selection. J166 *cagXY* was created in a similar fashion, but in two steps, first deleting *cag1-6*
381 with a chloramphenicol resistance cassette (*cat*) and selection on chloramphenicol, and then
382 deleting *cag9-25* with a kanamycin cassette, leaving only *cagX*, *cagY* (and its promoter), and
383 *cagA*, which in strain J166 is not on the *cag*PAI (56). J166 *cagY* was made in a series of 3
384 steps. First an unmarked deletion of *cag1-6* was constructed using contraselection. The region
385 was replaced by a *cat-rpsL* casette, resulting in streptomycin sensitive (*rpsL* encodes dominant
386 streptomycin sensitivity) and chloramphenicol resistant transformants. Then upstream and
387 downstream fragments were stitched together and the PCR product was used to replace the
388 cassette, leaving an unmarked deletion. Next, *cag9-25* were deleted as in the *cagXY* construct,
389 and replaced with a kanamycin cassette. Finally, contraselection was again used to excise the
390 *cagX* gene, bringing 313 bp upstream of *cagX* (putatively containing its promoter) immediately
391 upstream of *cagY*.

J166Δ*cagY*$_{MRR}$, with an in-frame markerless deletion of the MRR, was constructed using modifications of contraselection described previously (21). Briefly, the MRR was first replaced by insertion of the *cat-rpsL* cassette in streptomycin resistant *H. pylori* J166. Fragments upstream and downstream of the MRR were then each amplified with overlapping primers that permitted stitching of the two products in a second PCR reaction. The stitched product was ligated into pBluescript and used in a second transformation reaction to replace *cat-rpsL*, with selection on streptomycin. All *H. pylori* deletion mutants were sequence verified to confirm the correct construction.

Microfluidic adhesion assay

Microfluidic adhesion assays were assembled as previously described (57). In brief, 25 mm diameter, #1.5 glass coverslips were piranha etched to remove organic molecules and treated with 1% 3-aminopropyltriethoxysilane to add aminosilane groups. Recombinant human $\alpha_4\beta_1$, $\alpha_5\beta_1$ or $\alpha_L\beta_2$ integrin (R&D Systems, Minneapolis, MN) was adsorbed at 10 mg/liter concentration overnight at 4^{o}C resulting in approximately 2000 sites/μm^2. Coverslips were then washed and blocked with Hank's balanced salt solution with 0.1% human serum albumin. Where indicated, the blocking solution was supplemented with 5 mg/liter of anti-integrin β_1 blocking antibody P5D2 (Abcam, San Francisco, CA), low affinity locking anti-integrin β_1 antibody SG19, high affinity locking anti-integrin β_1 antibody TS2/16 (both from Biolegend, San Diego, CA), isotype control antibody B11/6 (Abcam, San Francisco, CA), or 2 mM $MnCl_2$ (Mn^{2+}) to activate integrin. Custom multi-channel microfluidic device (57) was vacuum sealed, outlets were attached to Exigo pumps to provide the negative pressure necessary to induce shear, and inlet reservoirs were loaded with *E. coli* or *H. pylori*. Prior to loading, liquid cultured bacteria were stained at an OD_{600} of 0.8 with 2% Vybrant DiI, DiD or DiO Cell-Labeling Solution (Grand Island, NY) in Brucella broth for 20 min at 37°C in the dark. Stained bacteria were washed twice with PBS and then resuspended in Brucella broth to the desired final OD_{600}. Competitive binding

assays were performed by mixing differently labeled WT and mutant bacteria at an OD_{600} of 0.4 (total OD_{600} 0.8). Shear was induced at 1 dyne/cm^2 for 3 minutes followed by a 3 minute period of no shear incubation to allow attachment. Then shear was increased to 1 dyne/cm^2 and 10 second videos were taken along the centerline of the channel in four field of views using an inverted TIRF research microscope (Nikon) equipped with a 60X numerical aperture 1.5 immersion TIRF objective and a 120 W arc lamp to capture epi-fluorescence images with the appropriate filter sets (488 nm for DiO, 510 nm for DiD and 543 nm for DiI). Images were captured using a 16-bit digital complementary metal oxide semiconductor Zyla camera (Andor, Belfast BT12 7AL, UK) connected to a PC (Dell) with NIS Elements imaging software (Nikon, Melville, NY). Images were collected with 2x2 binning at a resolution of 1024 x 1024 at a rate of 2 frames per second. Adherent bacteria were identified by the presence of fluorescence, which was cross-checked with an overlaid brightfield image to eliminate fluorescent noise. Small numbers of bacteria that were unstained (typically ~10%) were not counted. Bacteria that remained stationary or tethered after 10 seconds were counted visually in 3 fields of view, and the results were averaged for each biological replicate. To assess reliability, two observers (one blinded) independently scored adherent bacteria at 488 nm and 543 nm in 9 fields of view that contained competitive binding assays (WT and mutant). Mean similarity for the 18 observations was 0.94, which was calculated as $1-[|O_1 - O_2|/ \frac{1}{2}(O_1 + O_2)]$, where O_1 and O_2 are the independent scores for the two observers, and a value of 1.0 indicates perfect agreement. Data on integrin binding are representative of at least three biological replicates, which in most cases examined three fields of view in duplicate technical replicates.

Sequencing of *cagY*

The DNA sequences of *cagY* from *H. pylori* PMSS1 and KUS13A and B were determined using single molecule real-time sequencing (Pacific Biosciences, Menlo Park, CA). Briefly, *cagY* was amplified as previously described (21) and purified PCR products were

444 submitted to the DNA Technologies Core at the UC Davis Genome Center. The amplicons were
445 sequenced using a PacBio RSII sequencer, with P6C4 chemistry. Data were analyzed using
446 PacBio's SMRTportal Analysis 2.3.0. Sequences were deposited in GenBank and are available
447 under accession numbers KY613376 - KY613380. *cagY* sequences of *H. pylori* J166 were
448 previously published (21).
449
450 **Assessment of protein expression by fluorescence microscopy**
451 Liquid cultures of *H. pylori* or IPTG-induced *E. coli* strains were centrifuged (3,000 x g, 3
452 min) and resuspended in blocking buffer (PBS with 1% bovine serum albumin and 0.05%
453 Tween 20) at an OD_{600} of 0.4. Each culture was spotted on to two microscope slides using
454 cytofunnels in a cytospin at 1000 rpm for 15 min. Air dried slides were incubated for 1 h with
455 blocking buffer in a humid chamber followed by 1 h incubation with anti-*H. pylori* CagY MRR
456 antibody (31) diluted 1:1000 or anti-Yersinia invasin antibody (58) diluted 1:5000 in blocking
457 buffer. Slides were washed 3 times with PBS and incubated for 1 h in the dark with Alexa Fluor
458 488 goat anti-rabbit IgG (R37116, Life Technologies) diluted 1:10 in blocking buffer. After further
459 washing, the slides were mounted with FluoroShield with DAPI (Sigma). The slides were stored
460 in the dark and imaged the next day. Photos of all slides were captured with the same exposure
461 time for each antibody and DAPI. Fluorescence intensity was analyzed with the ImageJ
462 software, normalizing the total CagY fluorescence at a given threshold determined by the
463 positive WT sample to the area of the DAPI fluorescence of the bacterial particles.
464
465 **Immunoblots**
466 Expression of *E. coli* invasin and *H. pylori* CagY MRR were detected by electrophoresis
467 of lysates of liquid cultured bacteria as described previously (21), using polyclonal rabbit
468 antisera to invasin (1:15,000) or CagY MRR (1:10,000) as primary antibodies. Detection of
469 CagY expression in Δ$cagY_{MRR}$, an in-frame deletion of the MRR, was performed using antiserum

from rabbits immunized with the VirB10 portion at the C-terminus of CagY (1:1,000). To generate the antiserum, DNA encoding the C-terminus of *H. pylori* J166 CagY was PCR amplified (Table S1), cloned into pGEX-4T-3 vector and transformed into *E. coli* BL21 (both GE Healthcare). Expression of the GST-fusion protein and preparation of cell extracts was performed according to manufacturer's instructions. The GST-fusion protein was bound to Glutathione Sepharose 4B (GE Healthcare) in a column and the GST was cleaved off by thrombin. The eluate was run on a SDS-PAGE, the purified CagY C-terminus protein was cut out from the gel and was used to generate rabbit antisera according to standard protocols (Antibodies, Inc, Davis, CA).

IL-8 ELISA

IL-8 was measured essentially as described previously (59). Human AGS gastric adenocarcinoma cells (ATCC, Manassas, VA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 5% CO_2, 37°C. All antibiotics were excluded from the growth media 24 h prior to *H. pylori* co-culture. Approximately $5x10^5$ human AGS gastric adenocarcinoma cells were seeded in six well plates with 1.8 ml RPMI/10% fetal bovine serum, incubated overnight, and then co-cultured with bacteria diluted in 200 μl Brucella broth to give an MOI of 100:1. Brucella broth with no bacteria served as a baseline control. Supernatants were harvested after 20-22 hours of culture (37°C, 5% CO2), stored at -80°C, and then diluted 1:8 prior to IL-8 assay by ELISA (Invitrogen, Camarillo, CA) performed according to the manufacturer's protocol. WT *H. pylori* J166 or PMSS1 and its isogenic *cagY* deletion were included on every plate as positive and negative controls, respectively. IL-8 values were normalized to WT *H. pylori* determined concurrently.

High resolution field-emission gun scanning electron microscopy analyses

Bacteria were cultured alone or with AGS cells for 4 hrs at an MOI of 100:1. Bacteria were prepared for scanning electron microscopy as previously described (27, 60). Briefly, samples were cultured on poly-L-lysine coated glass coverslips, and fixed for 1 hour with 2.0% paraformaldehyde/2.5% gluteraldehyde in 0.05 M sodium cacodylate buffer. Cells were washed three times in 0.05 M sodium cacodylate buffer before secondary fixation with 0.1% osmium tetroxide for 15 minutes. Three additional 0.05 M sodium cacodylate buffer washes were performed before subjecting the samples to sequential ethanol dehydration. Cells were dried at the critical point and carbon-coated before imaging with an FEI Quanta 250 FEG-SEM. Pili were enumerated in a blinded fashion using ImageJ software.

Statistical analysis

Data are reported as mean ± SEM. Multiple groups were compared using ANOVA, with Tukey's or Bonferroni's post hoc test, or with Dunnett's post hoc test when compared only to WT. Two group comparisons were performed using Student's *t* test. All analyses were carried out using GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA). A P-value <0.05 was considered statistically significant.

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733

FIGURE LEGENDS

Fig 1. Microfluidic detection of bacterial adherence to recombinant $\alpha_5\beta_1$ integrin. (A) Schematic diagram and photograph of the microfluidic flow cell assembly. (B) Immunofluorescent detection of InvA on the surface of non-permeabilized IPTG-treated *E. coli* containing the pRI253 plasmid with or without *invA*. (C) Attachment to $\alpha_5\beta_1$ integrin of IPTG-treated *E. coli*. Each strain was used at an OD_{600} of 0.8, labeled with DiD or DiO and assayed separately. (D) Micrograph of *H. pylori* J166 labeled with DiO membrane dye, attached to $\alpha_5\beta_1$ integrin in the microfluidic flow cell. Brightfield and fluorescence overlay of the field of view (FOV) demonstrates fluorescent labeling of *H. pylori*.

Fig 2. $\alpha_5\beta_1$ integrin adherence of WT *H. pylori* J166 and *cag*PAI deletion mutants. (A) Adherent *H. pylori* J166 and PMSS1 per FOV as a function of bacterial optical cell density at 600 nm (OD_{600}). (B) Adherent *H. pylori* after pre-incubation of flow cells with B11/6 isotype control antibody, P5D2 antibody to sterically inhibit β_1 integrin binding, or antibodies to lock the integrin in the low (SG19) or high (TS2/16) affinity conformation, respectively. Treatment of integrin with Mn^{2+} to stabilize the high affinity state produced results similar to treatment with TS2/16 and the B11/6 isotype control antibody. (C) Adherence to $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins. (D) Adherence to $\alpha_5\beta_1$ integrin of J166 WT and deletion mutants, which were fluorescently labeled with DiO and DiI, respectively, mixed in a 1:1 ratio, and enumerated by counting fluorescent bacteria per field of view (FOV). Results are expressed as the ratio of deletion mutant to WT. (E) Steric inhibition with P5D2 antibody (white bars) demonstrated that adherence is integrin-specific. Δ*cagY* adherence was similar with and without steric inhibition, suggesting that it represents only non-specific background binding. Results are mean (± SEM) of 3 to 5 independent experiments. *P<0.05, **P<0.01, ***P<0.001.

759 **Fig 3. Schematic diagram of the *H. pylori* J166 *cag*PAI in the WT and selected deletion**
760 **mutants.** In J166Δ*cagY*, the entire *cagY* gene is replaced by a *cat-rpsL* cassette (streptomycin
761 susceptibility and chloramphenicol resistance). Δ$cagY_{MRR}$ has an unmarked, in-frame deletion of
762 the MRR created by contraselection. In J166 *cagXY*, *cag1-6* is replaced with *cat*, and *cag9-25* is
763 replaced with a kanamycin resistance cassette, starting from after the putative *cagY* promoter in
764 *cag9*. J166 *cagY* has an unmarked deletion of *cag1-6* and *cagX*, while *cag9-25* downstream of
765 the *cagX/Y* promoter in cag9 are replaced with a kanamycin resistance cassette. *cagA* is intact
766 in all strains since it is not on the *cag*PAI in J166 (56).
767
768 **Fig 4. Field emission scanning electron microscopy (FEG-SEM) of *H. pylori* demonstrates**
769 **that host cell contact is required for T4SS pilus formation.** (A) FEG-SEM of *H. pylori* J166
770 WT and Δ*cag*PAI cultured with or without AGS cells. Pili are indicated by white arrows. Scale
771 bar, 1 μm. (B) Enumeration of pili per bacterial cell. ***P<0.001, compared with WT.
772
773 **Fig 5. The CagY middle repeat region (MRR), but not the T4SS pilus, is required to bind**
774 **$\alpha_5\beta_1$ integrin in a host cell-free system and is expressed on the bacterial surface.** (A and
775 B) Immunoblot detection of the CagY MRR and VirB10 region in bacterial lysates. (C and D)
776 Immunofluorescent detection of the CagY MRR on the surface of non-permeabilized *H. pylori*.
777 (E and F) Flow channel competitive adherence to $\alpha_5\beta_1$ integrin. Results are expressed as the
778 ratio of deletion mutant to WT adherence, and represent the mean (± SEM) of at least 3
779 independent experiments. ***P<0.001
780
781 **Fig 6. Variation in the amino acid motif structure of the CagY MRR regulates T4SS**
782 **function by altering $\alpha_5\beta_1$ integrin binding.** Western blot detection of the CagY MRR in whole
783 cell bacterial lysates of *H. pylori* J166 (A) or PMSS1 (C) isogenic strains, each bearing unique
784 *cagY* alleles, or their Δ*cagY* deletion mutants. The corresponding amino acid structure of the

MRR is shown schematically as a series of A (orange) or B (yellow) motifs, each 31-39 residues, based on DNA sequence analysis as described previously (61). IL-8 induction (white bars) and integrin adhesion (black bars) relative to WT are shown for *H. pylori* J166 (B) and PMSS1 (D) that correspond to strains shown in panels A and C, respectively. (E) Western blot detection and schematic of the CagY MRR (derived as in panels A and C) from KUS13A, KUS13B, and isogenic variants in which *cagY* was deleted (Δ*cagY*) or replaced with that from the variant strain (i.e., KUS13A with $cagY_{13B}$ or KUS13B with $cagY_{13A}$). (F) IL-8 induction (white bars) and integrin adhesion (black bars) relative to WT for the strains shown in panel E. Quantitative results represent the mean (± SEM) of at least 3 independent experiments. *P<0.05, ***P<0.001 for comparison of WT to isogenic *cagY* deletion and variants. Results for IL-8 are adapted from references 20 and 22.

Fig 7. Recombination in *cagY* does not change its surface expression. (A) Immunofluorescent detection of the CagY MRR on the surface of non-permeabilized *H. pylori* PMSS1 with distinct *cagY* alleles from mouse output strains. (B) Quantification of CagY MRR mean fluorescence intensity (MFI) normalized to DAPI. Results are expressed as the percentage ratio of deletion mutant to WT, and represent the mean (± SEM) of 3 independent experiments. *P<0.05, compared to WT.

SUPPORTING FIGURE LEGENDS

S1 Fig. Adherence of invasin expressing *E. coli*. (A) Immunoblot of *E. coli* with plasmid pRI253 with or without *invA*, untreated or treated with IPTG to induce InvA expression. Full length InvA is around 100 kDa and the lower bands represent degradation products (55, 58). (B) Attachment to $\alpha_5\beta_1$ integrin in a competitive assay; the two strains of *E. coli* were stained with either DiD or DiO and mixed 1:1 for a total OD_{600} of 0.8. A parallel dye swap was performed to exclude influence of the dye on the integrin attachment.

S2 Fig. Fluorophore labeling control experiments. (A) Percent of cells stained with DiI or DiO ([fluorescently stained cells divided by total cells seen on brightfield] x 100) was determined for *H. pylori* J166 WT, Δ*cag*PAI, Δ*cagY*, and isogenic strains expressing functional (Δ*cagY*[Out1]) or non-functional (Δ*cagY*[Out3] *cagY* alleles. (B) *H. pylori* J166 WT and Δ*cag*PAI (each at OD_{600}=0.4) were labeled with DiI or DiO, respectively (left), mixed 1:1, and imaged with a 546 nm (DiI) or 488 nm (DiO) light source. Parallel experiments were performed with a dye swap (middle). WT labeled with DiI was also mixed with WT labeled with DiO (right). Binding to $\alpha_5\beta_1$ integrin was similar whether detected with DiO or DiI fluorescent dyes, and whether in competition with a strain with low or high binding ability. Results for WT measured in a competition setting were also very similar to that when measured individually (compare with Fig 2B). Data represent mean ± SEM of ≥ 3 experiments.

S3 Fig. Expression of CagY in wild type and isogenic knockouts of *H. pylori* J166. (A) Immunoblot of CagY in WT *H. pylori* and isogenic knockouts of *cagY*, *cagA*, *cagI/L*, and *cagE*. Immunoblot of UreB is shown as a loading control. (B-D) Quantification of whole cell CagY MRR fluorescence signal normalized to DAPI in non-permeabilized WT *H. pylori* and isogenic knockouts or *cagY* variants. CagY surface expression in strains with MRR motifs that bind $\alpha_5\beta_1$

integrin and have a functional *cag*PAI (variants 1 and 2) is similar to that in strains that do not bind integrin and do not have a functional *cag*PAI (variants 3 and 4), though all are generally lower than J166 WT. Results are expressed as the percentage ratio of deletion mutant to WT, and represent the mean (± SEM) of 3 independent experiments. (MFI=mean fluorescence intensity) *P<0.05, **P<0.01, ***P<0.001 compared to WT.

S4 Fig. Field emission scanning electron microscopy (FEG-SEM) of *H. pylori* PMSS1. (A) FEG-SEM of *H. pylori* PMSS1 WT and Δ*cag*PAI cultured with or without AGS cells. Pili are indicated by white arrows. (B) Enumeration of pili per bacterial cell. ***P<0.001.

S5 Fig. T4SS function in various *cag*PAI *H. pylori* J166 mutants.

IL-8 induction in AGS cell co-cultured with J166 WT, Δ*cagY*, *cagXY*, *cagY* and Δ$cagY_{MRR}$. ***P<0.001.

S1 Table. Primers used for PCR.

Table 1. *H. pylori* strains

Strain	Relevant characteristic(s)	Antibiotic resistance[a]	Source (Reference)
E. coli strains			
BL21 *invA* +	*E. coli* BL21 with plasmid pRI253	Amp	(55)
BL21 *invA* +	*E. coli* BL21 with plasmid pRI253Δ*invA*	Amp	this study
H. pylori wild type strains			
J166	Wild type		(62)
PMSS1	Wild type		(38)
KUS13A	Clinical isolate from patient KUS13		(63)
KUS13B	Isolate from patient KUS13 7.4 yrs after isolate A		(63)
Deletion mutants			
J166Δ*cag*PAI	Deletion of the entire *cag*PAI	Cm, Km	(39)
J166Δ*cagA*	J166Δ*cagA*::*aphA*	Km	this study
J166Δ*cagI/L*	J166Δ*cagI/L*::*aphA*	Km	this study
J166Δ*cagY*	J166 Str^RΔ*cagY*::*cat_rpsL*	Cm	(21)
J166Δ*cagE*	J166Δ*cagE*::*aphA*	Km	this study
J166 *cagXY*	J166Δ*cag1-6*::*cat,*Δ*cag9-25*::*aphA*	Cm, Km	this study
J166 *cagY*	J166Δ*cag1-6,* Δ*cag8,* Δ*cag9-25*::*aphA*	Km	this study
J166Δ$cagY_{MRR}$	J166 Str^RΔ$cagY_{MRR}$	Str	this study
J166 *cagY* replacements			
Δ*cagY*[mOut1]	J166Δ*cagY* replaced with *cagY* from mOut1	Str	(21)
Δ*cagY*[mOut2]	J166Δ*cagY* replaced with *cagY* from mOut2	Str	(21)
Δ*cagY*[mOut3]	J166Δ*cagY* replaced with *cagY* from mOut3	Str	(21)
Δ*cagY*[mOut4]	J166Δ*cagY* replaced with *cagY* from mOut4	Str	(21)
PMSS1 *cagY* replacements			
Δ*cagY*[Out1]	PMSS1Δ*cagY* replaced with *cagY* from Out1	Str	(23)
Δ*cagY*[Out2]	PMSS1Δ*cagY* replaced with *cagY* from Out2	Str	(23)
Δ*cagY*[Out3]	PMSS1Δ*cagY* replaced with *cagY* from Out3	Str	(23)
CagY replacement clinical isolates			
KUS13AΔ*cagY*	KUS13AΔ*cagY*::*cat_rpsL*	Cm	(23)
KUS13BΔ*cagY*	KUS13BΔ*cagY*::*cat_rpsL*	Cm	(23)
KUS13AΔ*cagY*[KUS13B]	KUS13AΔ*cagY* replaced with *cagY* from KUS13B	Str	(23)
KUS13BΔ*cagY*[KUS13A]	KUS13BΔ*cagY* replaced with *cagY* from KUS13A	Str	(23)

[a]Amp, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Str, streptomycin