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RESEARCH ARTICLE

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 (*cag*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, although 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

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acts like a molecular rheostat that alters T4SS function and modulates the host immune response to promote persistent infection.

KEYWORDS CagA, CagY, *Helicobacter pylori*, integrin, pathogenicity island, type IV secretion system

Helicobacter pylori infection most often causes only asymptomatic gastritis, but *H. pylori* 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), an ~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 β_1 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, although 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 gamma interferon (IFN-γ) 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

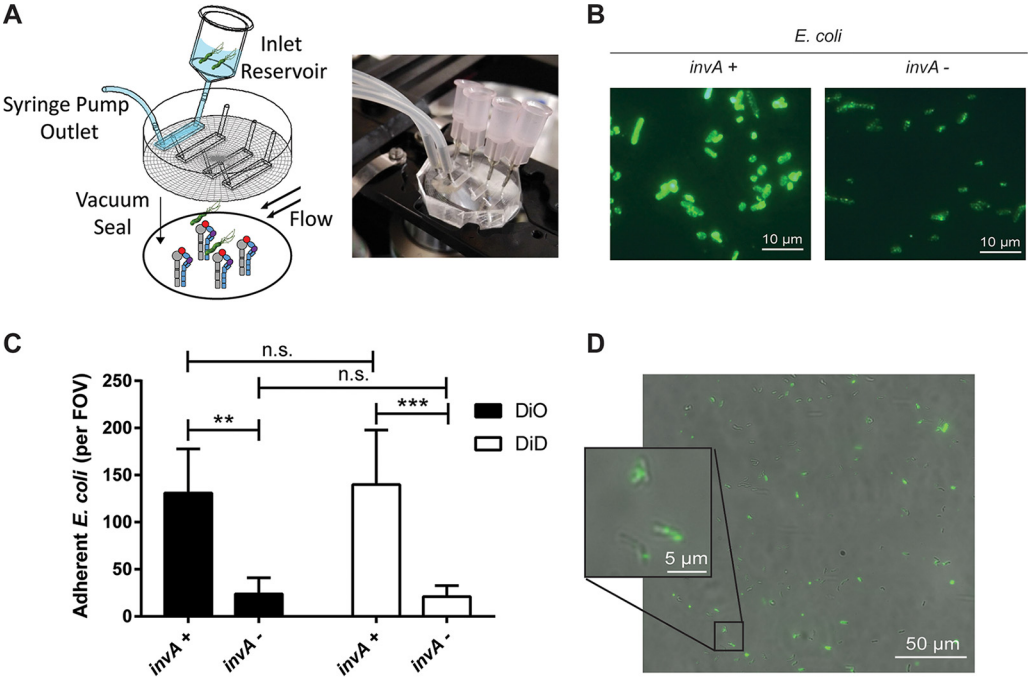

FIG 1 Microfluidic detection of bacterial adherence to recombinant $\alpha_5\beta_1$ integrin. (A) Schematic diagram and photograph of the microfluidic flow cell assembly. (B) Immunofluorescent detection of InvA on the surface of nonpermeabilized IPTG-treated *E. coli* cells containing the pRI253 plasmid 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. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant. (D) Micrograph of *H. pylori* J166 labeled with DiO membrane dye, attached to $\alpha_5\beta_1$ integrin in the microfluidic flow cell. Bright-field and fluorescence overlay of the field of view (FOV) demonstrates fluorescent labeling of *H. pylori*.

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 that contains a VirB10 domain that is an essential part of a complete T4SS structure and an MRR region that mediates close contact with the host cell and modulates T4SS function.

RESULTS

***H. pylori* binds to $\alpha_5\beta_1$ 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 colocalization to β_1 integrin on host cells *in vitro* (11, 12). To demonstrate binding of intact live *H. pylori* cells to β_1 integrin, we developed a microfluidic assay in which human recombinant $\alpha_5\beta_1$ integrin was coated onto glass coverslips, 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 (isopropyl-β-D-thiogalactopyranoside) stimulation (see Fig. S1A in the supplemental material) 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 $\alpha_5\beta_1$ integrin 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

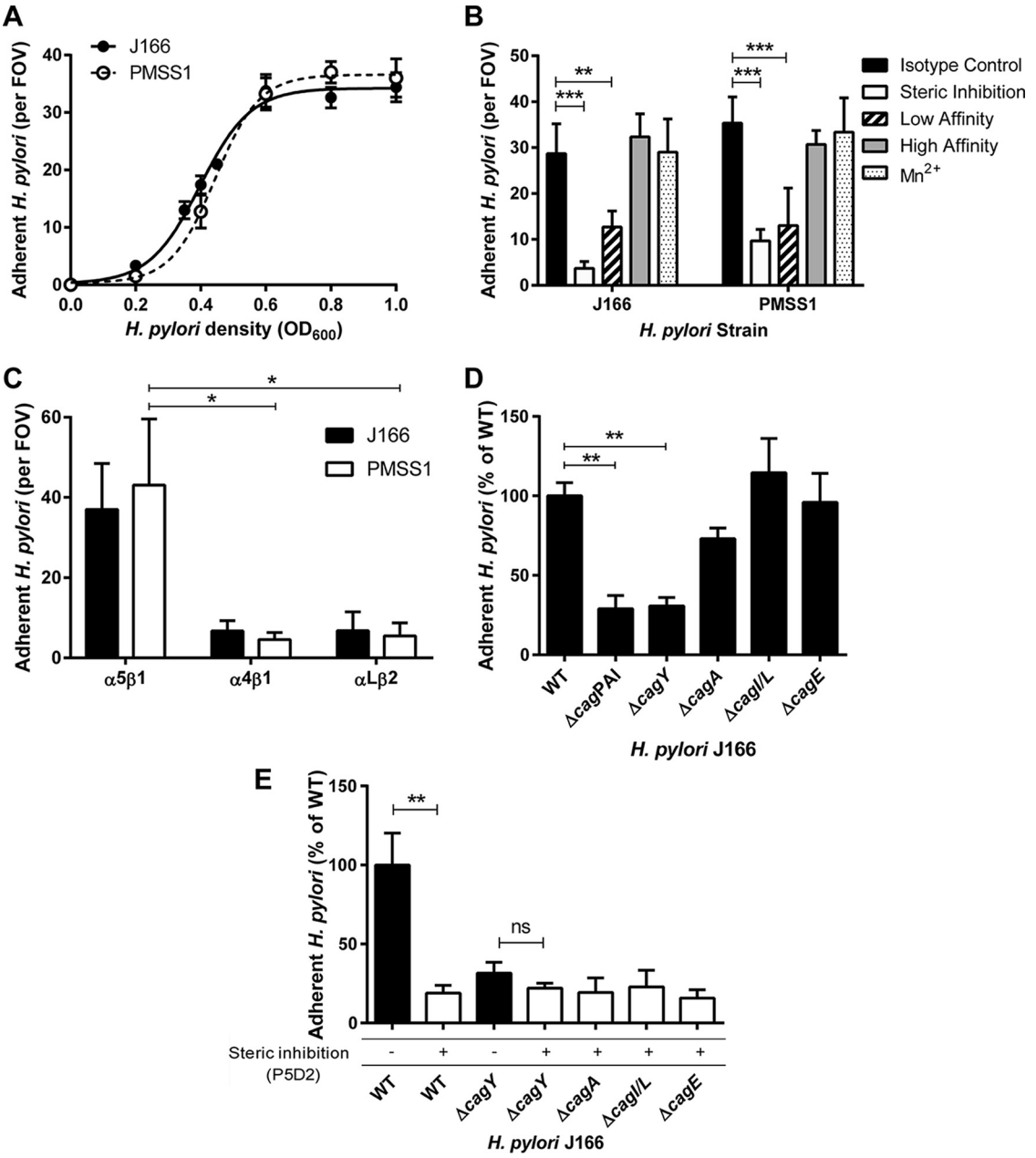

FIG 2 $\alpha_5\beta_1$ integrin adherence of WT *H. pylori* J166 and *cag*PAI deletion mutants. (A) Adherent *H. pylori* J166 and PMSS1 cells per field of view (FOV) as a function of bacterial optical cell density at 600 nm (OD_{600}). (B) Adherent *H. pylori* after preincubation of flow cells with B11/6 isotype control antibody, P5D2 antibody to sterically inhibit β_1 integrin binding, or antibodies to lock the integrin in the low-affinity (SG19) or high-affinity (TS2/16) 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 the 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 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* mutant adherence was similar with and without steric inhibition, suggesting that it represents only nonspecific background binding. Results are the mean ± SEM from 3 to 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

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* cells were 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 at 600 nm (OD_{600}) of 0.8 (Fig. 2A). This correlates with approximately 4×10^8 bacterial cells per

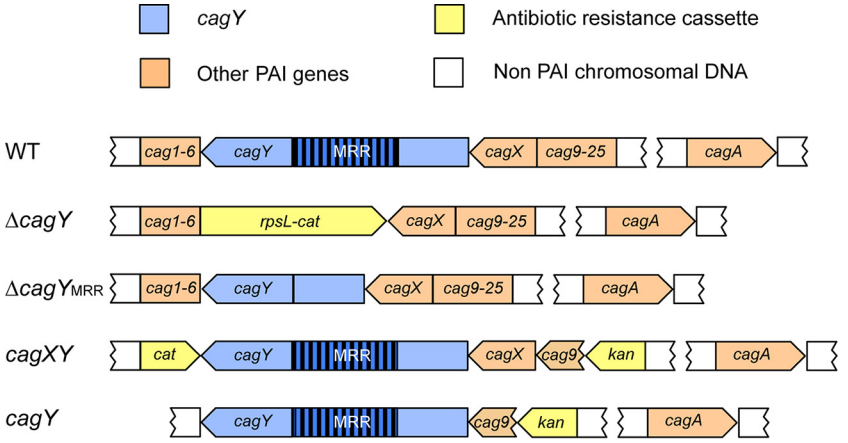

FIG 3 Schematic diagram of the *H. pylori* J166 *cag*PAI in the WT and selected deletion mutants. In J166 Δ*cagY*, the entire *cagY* gene is replaced by a *cat-rpsL* cassette (coding for streptomycin susceptibility and chloramphenicol resistance). The $\Delta cagY_{MRR}$ mutant has an unmarked, in-frame deletion of the MRR created by contraselection. In J166 *cagXY*, *cag1* to *6* are replaced with *cat*, and *cag9* to *25* are replaced with a kanamycin resistance cassette, starting from after the putative *cagY* promoter in *cag9*. J166 *cagY* has an unmarked deletion of *cag1* to *6* and *cagX*, while *cag9 to 25* downstream of the *cagX/Y* promoter in *cag9* are replaced with a kanamycin resistance cassette. *cagA* is intact in all strains since it is not on the *cag*PAI in J166 (56).

ml and was used for all subsequent experiments. Binding was blocked by preincubating the integrin-coated coverslips 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 preincubation with SG19 antibody decreased *H. pylori*-integrin binding, while the TS2/16 antibody, which 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 that 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* cells per field of view (FOV) was determined, and the results were analyzed as the percentage of adherence of the mutant compared to WT. Initial control experiments demonstrated that WT and selected mutant strains stained with similar efficiency with both dyes (see Fig. S2A in the supplemental material), and levels of adhesion were independent of the dye and were 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 that of the Δ*cag*PAI mutant (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 immunoblotting (see Fig. S3A in the supplemental material). The Δ*cagY* mutant 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.

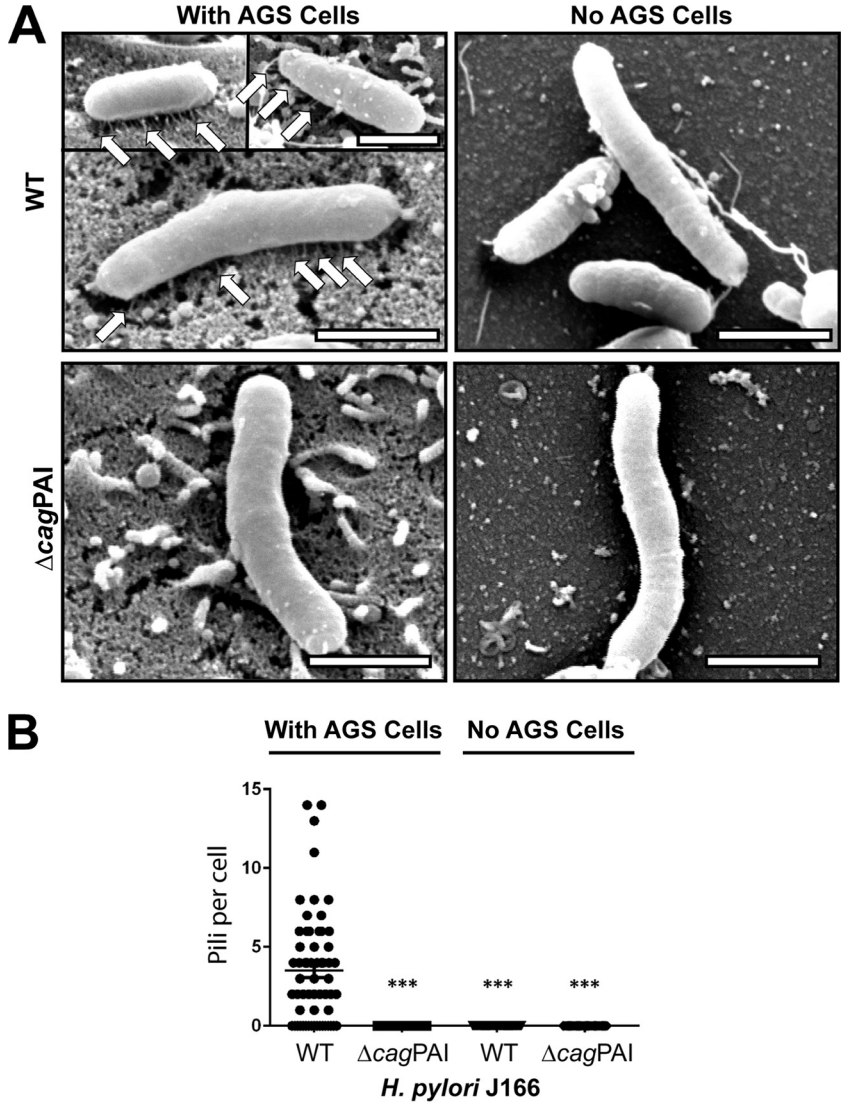

FIG 4 Field emission gun scanning electron microscopy (FEG-SEM) of *H. pylori* demonstrates that host cell contact is required for T4SS pilus formation. (A) FEG-SEM of the *H. pylori* J166 WT and Δ*cag*PAI mutant cultured with or without AGS cells. Pili are indicated by white arrows. Scale bar, 1 μm. (B) Enumeration of pili per bacterial cell. ***, $P < 0.001$, compared with WT.

CagY-mediated integrin binding is independent of the T4SS pilus. *H. pylori* T4SS pilus formation is thought to require host cell contact (27), although this has never been formally demonstrated. Since *H. pylori* attachment to integrin in the flow channel occurs in the absence of host cells, this suggests that *H. pylori* can bind to $\alpha_5\beta_1$ integrin independent of the T4SS pilus. To examine this further, we used field emission gun scanning electron microscopy (FEG-SEM) to image the T4SS pili in the *H. pylori* WT and Δ*cag*PAI mutant, cocultured with or without AGS gastric epithelial cells. Numerous pili were observed on WT *H. pylori* J166, but only in the presence of AGS cells (Fig. 4). As expected, no pili were detected on J166 Δ*cag*PAI. The same results were found for the *H. pylori* PMSS1 WT and Δ*cag*PAI mutant (see Fig. S4 in the supplemental material). Culture of *H. pylori* together with $\alpha_5\beta_1$ integrin also failed to induce pilus formation (data not shown). Therefore, under shear flow in this cell-free system, CagY-mediated binding to $\alpha_5\beta_1$ integrin does not require formation of the T4SS pilus. To further demonstrate that CagY is sufficient for integrin binding in the absence of the T4SS pilus, all of the PAI genes were deleted, except *cagX* and *cagY*, which are transcribed as an operon from a putative promoter located in *cag9*, upstream of *cagX* (28, 29). This

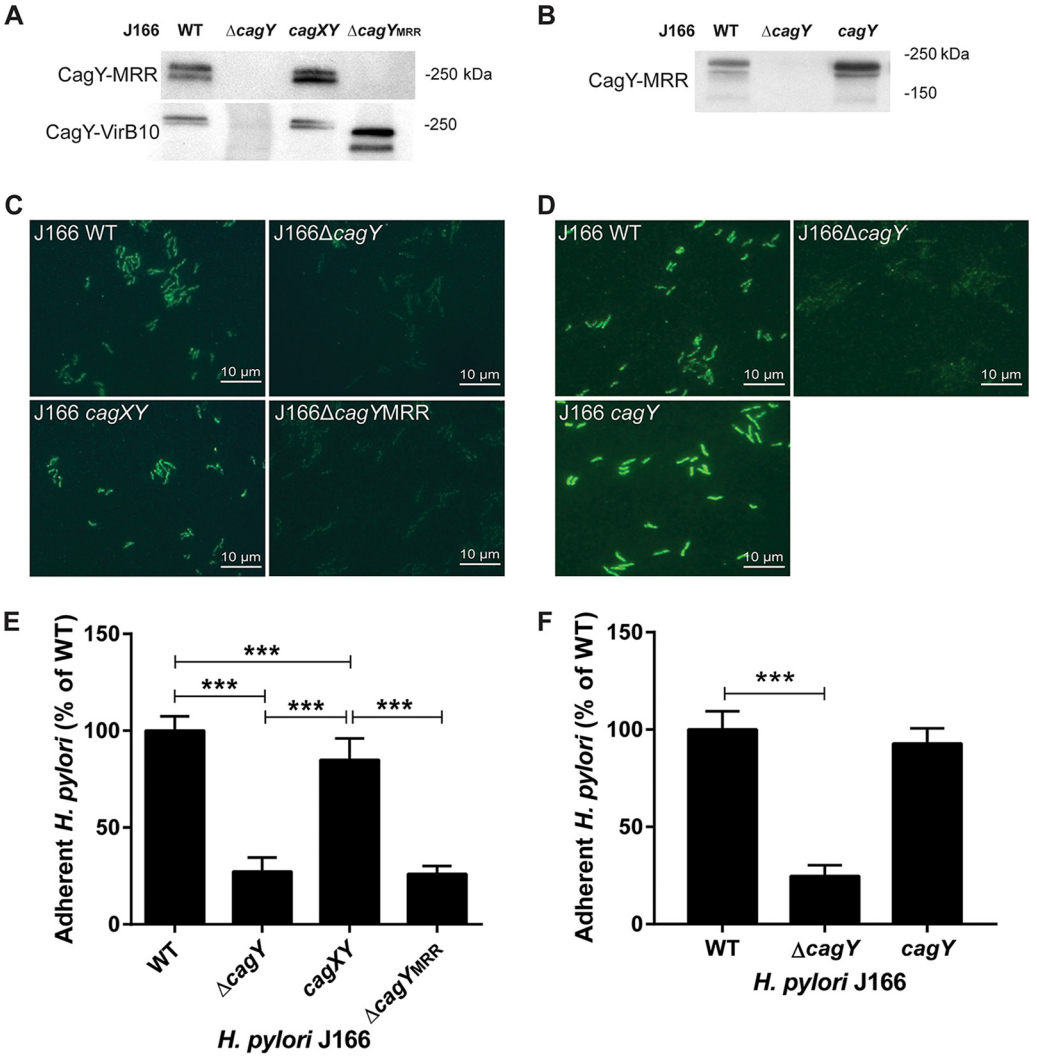

FIG 5 The CagY middle repeat region (MRR), but not the T4SS pilus, is required to bind $\alpha_5\beta_1$ integrin in a host cell-free system and is expressed on the bacterial surface. (A and B) Immunoblot detection of the CagY MRR and VirB10 region in bacterial lysates. (C and D) Immunofluorescent detection of the CagY MRR on the surface of nonpermeabilized *H. pylori*. (E and F) Flow channel competitive adherence to $\alpha_5\beta_1$ integrin. Results are expressed as the ratio of deletion mutant to WT adherence and represent the mean ± SEM from at least 3 independent experiments. ***, $P < 0.001$.

mutant, designated *cagXY*, is shown schematically in Fig. 3 compared to the J166 WT and Δ*cagY* mutant. J166 *cagXY* expresses CagY on the bacterial surface (Fig. 5A and C) but fails to induce a robust IL-8 response in AGS cells due to the lack of a T4SS (see Fig. S5 in the supplemental material). In the flow channel $\alpha_5\beta_1$ integrin binding assay, J166 *cagXY* binds at a level similar to the J166 WT (Fig. 5E). To exclude a role for CagX, we deleted all *cag*PAI genes and stitched *cagY* directly to the promoter in *cag9*, creating J166 *cagY*. Similar to J166 *cagXY*, J166 *cagY* fails to induce IL-8 (Fig. S5), but expresses CagY and binds to $\alpha_5\beta_1$ integrin similarly to the J166 WT (Fig. 5B and D and F). Together these results suggest that in this assay, *H. pylori* binds to $\alpha_5\beta_1$ integrin predominantly via a CagY-dependent mechanism, but independently of T4SS pilus formation. This conclusion is also supported by the observation that integrin binding in the J166 Δ*cagI/L* and Δ*cagE* mutants, which do not form a T4SS pilus (27), is similar to that in the 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 *cagA*, *cagI/L*, and *cagE* deletion mutants (Fig. S3B) and in J166 *cagXY* (Fig. 5A; Fig. 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 J166 Δ$cagY_{MRR}$), which is shown schematically in Fig. 3. J166 Δ$cagY_{MRR}$ 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, J166 Δ$cagY_{MRR}$ 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 nonhuman primate models, that recombination in the *cagY* MRR regulates T4SS function (21, 23), although 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 nonfunctional 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 (Fig. 6C and D). 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 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 F). 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 blotting 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 relationship to T4SS function or integrin binding (Fig. 7A). Analysis of fluorescence intensity normalized to DAPI (4′,6-diamidino-2-phenylindole) staining demonstrated quantitatively that CagY was expressed on the bacterial surface at similar levels, with no detection in the negative control (Fig. 7B). Quantitation of expression on the bacterial surface of isogenic *cag*PAI mutants of J166 similarly showed no relationship to T4SS function or integrin binding, although all MRR variants showed reduced expression (Fig. S3D), perhaps related to the reduction in the number of MRR motifs. These results suggest that changes in the motif structure of CagY on the bacterial

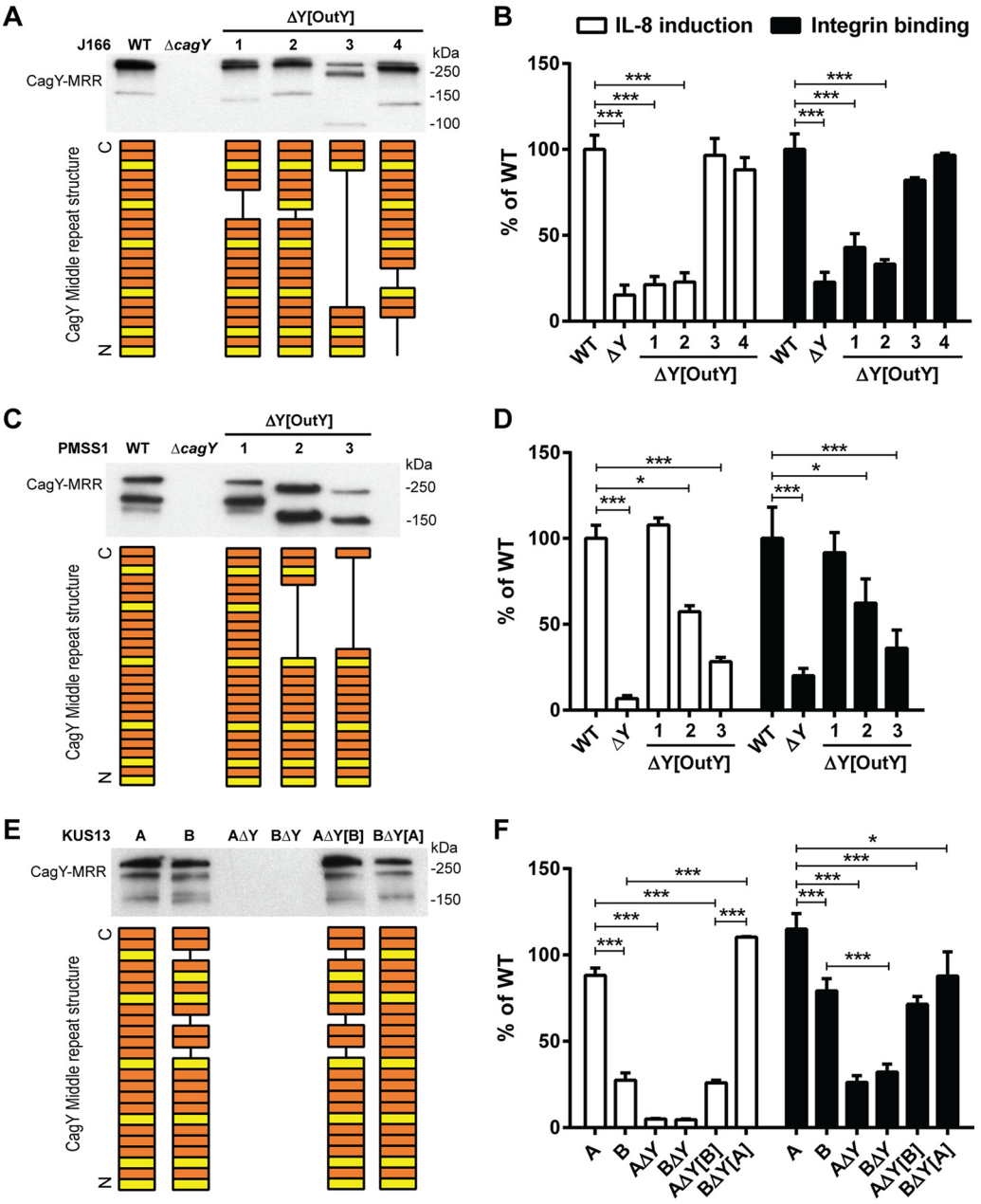

FIG 6 Variation in the amino acid motif structure of the CagY MRR regulates T4SS function by altering $\alpha_5\beta_1$ integrin binding. Shown is Western blot detection of the CagY MRR in whole-cell bacterial lysates of *H. pylori* J166 (A) or PMSS1 (C) isogenic strains, each bearing unique *cagY* alleles, 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 to 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), which 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 from at least 3 independent experiments. *, $P < 0.05$, and ***, $P < 0.001$, for comparison of the WT to the isogenic *cagY* deletion mutant and variants. Results for IL-8 are adapted from references 21 and 23.

surface modulate T4SS function by altering bacterial adhesion to $\alpha_5\beta_1$ integrin, rather than altering surface presentation of CagY.

DISCUSSION

H. pylori persistence in the gastric mucosa is often attributed to evasion of the innate and adaptive immune responses, including antimicrobial peptides (32), Toll-like recep-

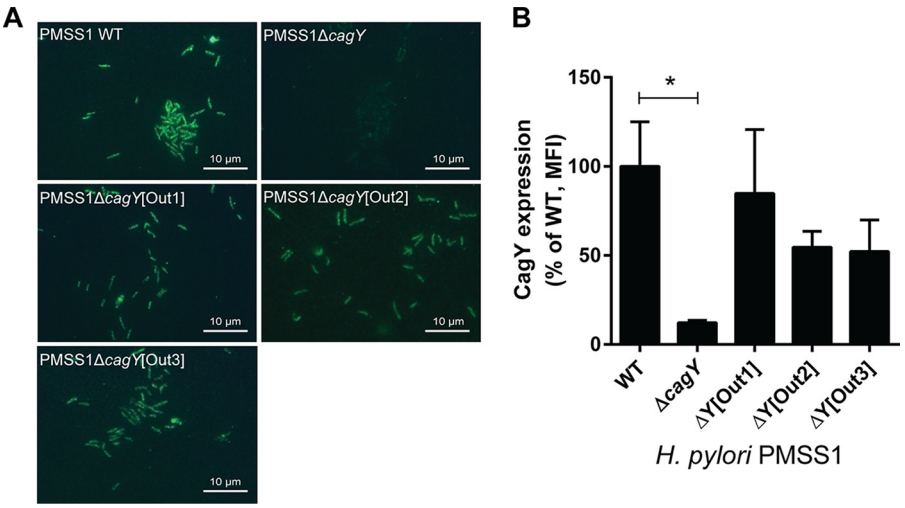

FIG 7 Recombination in *cagY* does not change its surface expression. (A) Immunofluorescent detection of the CagY MRR on the surface of nonpermeabilized *H. pylori* PMSS1 with distinct *cagY* alleles from mouse output strains. (B) Quantification of CagY MRR mean fluorescence intensity (MFI) normalized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

tor 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*, although 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 IFN-γ-dependent immune response (23). Thus, *cagY* recombination can modulate T4SS function and may be a bacterial strategy to both up- and down-regulate the host immune response to promote persistent infection.

Here we addressed the mechanism by which recombination in the MRR alters T4SS function. Since CagY is a ligand for $\alpha_5\beta_1$ integrin, which is essential for T4SS function, we hypothesized that changes in the amino acid motif structure from recombination in the MRR might alter integrin binding and modulate T4SS function. Analysis of whole bacterial cells in a microfluidic assay demonstrated CagY-dependent and integrin conformation-specific binding to $\alpha_5\beta_1$, which correlated closely with T4SS function in isogenic variants that differed only in the MRR region of CagY. This binding was

independent of the T4SS pilus, which was not formed under these cell-free conditions, although the MRR was expressed on the bacterial surface as described previously (31). Moreover, we could detect the MRR on the bacterial surface even when the entire *cag*PAI was deleted, except for *cagY* and the upstream promoter. Binding to $\alpha_5\beta_1$ integrin was not dependent upon CagA, CagE, or CagL, which was originally identified as the ligand for $\alpha_5\beta_1$ integrin (11). While CagL is clearly essential for T4SS function, more recent studies have suggested that it binds $\alpha_V\beta_6$ and $\alpha_V\beta_8$ integrin and not $\alpha_5\beta_1$ integrin (17). The results from yeast two-hybrid studies (12) also identified CagI as a β_1 integrin binding partner, which we could not confirm in whole bacterial cells.

Previous studies have found that the VirB10 ortholog at the C terminus of CagY bound to $\alpha_5\beta_1$ integrin, but not the MRR region. However, these studies examined protein-protein interactions by yeast two-hybrid assay and immunoprecipitation or by surface plasmon resonance (12, 46), which may not reflect binding in a whole bacterial cell. Since the isogenic *cagY* variants examined here differed only in the MRR, and deletion of the MRR eliminated $\alpha_5\beta_1$ integrin binding, our results suggest that the *H. pylori* MRR is required for binding to $\alpha_5\beta_1$ integrin in an intact bacterial cell. However, we have not directly examined MRR binding to $\alpha_5\beta_1$ integrin, so the MRR may not itself be an integrin ligand but instead may modulate binding of the VirB10 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 the WT and Δ*cag*PAI mutant (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 (Fig. 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, further enhancing integrin binding and injection of effector molecules. Such a scenario might entail MRR-dependent integrin signaling, including activation of focal adhesion kinase (FAK) and the Src family kinase, although others have shown that only the extracellular domains of the β_1 integrin are important for CagA translocation (12). On the other hand, it is logically possible that changes in the MRR affect integrin binding and T4SS function independently. Although the details remain to be elucidated, we hypothesize that CagY-dependent binding to $\alpha_5\beta_1$ integrin serves as a molecular rheostat that "tunes" the optimal balance between the competing pressures of gastric inflammation, which serves a metabolic function for the bacterium

TABLE 1 *E. coli* and *H pylori* strains used in this study

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

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

on the one hand, but comes at a cost of exposure to immune pressure, decreased bacterial load, and 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 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, pRI253 Δ*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 and cultured for an additional 2 to 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 the 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.

Construction of *H. pylori* mutants. Six *H. pylori* J166 mutants were constructed (Table 1). For J166 Δ*cagA*, J166 Δ*cagI/L*, and J166 Δ*cagE*, DNA fragments upstream and downstream of the respective gene deletion were PCR amplified using primers (see Table S1 in the supplemental material) with restriction sites that permitted ligation to a kanamycin resistance gene (*aphA*) and insertion into the multiple cloning site of pBluescript (Stratagene, La Jolla, CA). The resulting plasmid was transformed into *E. coli* TOP10 (Invitrogen) according to the manufacturers' instructions, and transformants were grown overnight on Luria-Bertani (LB) plates containing kanamycin. Resistant colonies were inoculated into selective

LB broth, and plasmids from the resulting culture were purified with a QIAprep spin miniprep kit (Qiagen). Plasmids were sequenced and digested with appropriate enzymes for verification of correct construction prior to natural transformation of *H. pylori* with kanamycin selection. J166 *cagXY* was created in a similar fashion, but in two steps: first deleting *cag1* to *6* with a chloramphenicol resistance cassette (*cat*) and selection on chloramphenicol and then deleting *cag9* to *25* with a kanamycin cassette, leaving only *cagX, cagY* (and its promoter), and *cagA*, which in strain J166 is not on the *cag*PAI (56). J166 *cagY* was made in a series of 3 steps. First an unmarked deletion of *cag1* to *6* was constructed using contraselection. The region was replaced by a *cat-rpsL* casette, resulting in streptomycin-sensitive (*rpsL* encodes dominant streptomycin sensitivity) and chloramphenicol-resistant transformants. Then, upstream and downstream fragments were stitched together, and the PCR product was used to replace the cassette, leaving an unmarked deletion. Next, *cag9* to *25* were deleted as in the *cagXY* construct and replaced with a kanamycin cassette. Finally, contraselection was again used to excise the *cagX* gene, bringing 313 bp upstream of *cagX* (putatively containing its promoter) immediately upstream of *cagY*.

J166 Δ$cagY_{MRR}$, 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. 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 no. 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 a 10-mg/liter concentration overnight at 4°C, resulting in approximately 2,000 sites/μm^2. Coverslips were then washed and blocked with Hanks' 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, Inc., San Francisco, CA), low-affinity locking anti-β_1 integrin antibody SG19, high-affinity locking anti-β_1 integrin antibody TS2/16 (both from BioLegend, San Diego, CA), isotype control antibody B11/6 (Abcam, Inc., San Francisco, CA), or 2 mM $MnCl_2$ (Mn^{2+}) to activate integrin. A custom multichannel 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 phosphate-buffered saline (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} of 0.8). Shear was induced at 1 dyne/cm^2 for 3 min followed by a 3-min period of no-shear incubation to allow attachment. Then, shear was increased to 1 dyne/cm^2, and 10-s videos were taken along the centerline of the channel in four field of views using an inverted total internal reflection fluorescence (TIRF) research microscope (Nikon) equipped with a 60× numerical aperture 1.5 immersion TIRF objective and a 120-W arc lamp to capture epifluorescence 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-b digital complementary metal oxide semiconductor Zyla camera (Andor, Belfast, United Kingdom) connected to a PC (Dell) with NIS Elements imaging software (Nikon, Melville, NY). Images were collected with 2-by-2 binning at a resolution of 1,024 by 1,024 at a rate of 2 frames per s. Adherent bacteria were identified by the presence of fluorescence, which was cross-checked with an overlaid bright-field 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 s were counted visually in 3 fields of view, and the results were averaged for each biological replicate. To assess reliability, two observers (one "blind") 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 $1 - [|O_1 - O_2|/1/2(O_1 + O_2)]$, 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 submitted to the DNA Technologies Core at the UC Davis Genome Center. The amplicons were sequenced using a PacBio RSII sequencer with P6C4 chemistry. Data were analyzed using PacBio's SMRTportal Analysis 2.3.0. *cagY* sequences of *H. pylori* J166 were previously published (21).

Assessment of protein expression by fluorescence microscopy. Liquid cultures of *H. pylori* or IPTG-induced *E. coli* strains were centrifuged (3,000 × *g*, 3 min) and resuspended in blocking buffer (PBS with 1% bovine serum albumin and 0.05% Tween 20) at an OD_{600} of 0.4. Each culture was spotted onto two microscope slides using Cytofunnels in a Cytospin centrifuge at 1,000 rpm for 15 min. Air-dried slides were incubated for 1 h with blocking buffer in a humid chamber followed by 1 h of incubation with anti-*H. pylori* CagY MRR antibody (31) diluted 1:1,000 or anti-*Yersinia* invasin antibody (58) diluted 1:5,000 in blocking buffer. Slides were washed 3 times with PBS and incubated for 1 h in the dark with Alexa Fluor 488 goat anti-rabbit IgG (R37116; Life Technologies, Inc.) diluted 1:10 in blocking buffer. After further washing, the slides were mounted with Fluoroshield with DAPI (Sigma). The slides were stored in

the dark and imaged the next day. Photos of all slides were captured with the same exposure time for each antibody and DAPI. Fluorescence intensity was analyzed with the ImageJ software, normalizing the total CagY fluorescence at a given threshold determined by the positive WT sample to the area of the DAPI fluorescence of the bacterial particles.

Immunoblots. Expression of *E. coli* invasin and *H. pylori* CagY MRR was detected by electrophoresis of lysates of liquid-cultured bacteria as described previously (21), using polyclonal rabbit antisera to invasin (1:15,000) or CagY MRR (1:10,000) as the primary antibodies. Detection of CagY expression in the Δ$cagY_{MRR}$ mutant, which contains 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 from GE Healthcare). Expression of the glutathione *S*-transferase (GST)-fusion protein and preparation of cell extracts were performed according to the 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 SDS-PAGE, and the purified CagY C-terminus protein was cut out from the gel and 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 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. All antibiotics were excluded from the growth media 24 h prior to *H. pylori* coculture. Approximately 5×10^5 human AGS gastric adenocarcinoma cells were seeded into six-well plates with 1.8 ml RPMI–10% fetal bovine serum, incubated overnight, and then cocultured with bacteria diluted in 200 μl brucella broth to give a multiplicity of infection (MOI) of 100:1. Brucella broth with no bacteria served as a baseline control. Supernatants were harvested after 20 to 22 h of culture (37°C, 5% CO_2), stored at −80°C, and then diluted 1:8 prior to IL-8 enzyme-linked immunosorbent assay (ELISA; Invitrogen, Camarillo, CA) performed according to the manufacturer's protocol. WT *H. pylori* J166 or PMSS1 and its isogenic *cagY* deletion mutant 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 h 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 h with 2.0% paraformaldehyde–2.5% glutaraldehyde 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 min. 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 blind fashion using ImageJ software.

Statistical analysis. Data are reported as the mean ± standard error of the mean (SEM). Multiple groups were compared using analysis of variance (ANOVA), with Tukey's or Bonferroni's *post hoc* test, or with Dunnett's *post hoc* test 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, Inc., San Diego, CA). A P value of <0.05 was considered statistically significant.

Accession number(s). Sequences have been deposited in GenBank and are available under accession no. KY613376 to KY613380.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00717-18.

FIG S1, TIF file, 1.3 MB.

FIG S2, TIF file, 1.9 MB.

FIG S3, TIF file, 3.6 MB.

FIG S4, TIF file, 4.2 MB.

FIG S5, TIF file, 0.1 MB.

TABLE S1, DOCX file, 0.1 MB.

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