

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

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25 Running title: Modulation of *H. pylori* T4SS by CagY-dependent alteration in integrin binding

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32

## ABSTRACT

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

50

## IMPORTANCE

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

62

## INTRODUCTION

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

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

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

91         It has long been known that passage of *H. pylori* in mice results in loss of T4SS function  
92 (19, 20). We previously demonstrated that this is typically a result of recombination events in  
93 *cagY* (21), a *virB10* ortholog that contains in its middle repeat region (MRR) an extraordinary  
94 series of direct DNA repeats that are predicted to encode in-frame insertions or deletions in a  
95 surface-exposed region of the protein (22). Recombination events in the *cagY* MRR lead to  
96 expression of an alternative CagY allele that can modulate T4SS function, including induction of  
97 IL-8 and translocation of CagA (21). This modulation can occur in a graded fashion, and cause  
98 both gain and loss of T4SS function (21). More recently, we demonstrated that IFN $\gamma$  and CD4+  
99 T cells are essential for *cagY*-mediated loss of T4SS function, which can rescue colonization in  
100 *IL10*<sup>-/-</sup> mice that have an exaggerated inflammatory response to *H. pylori* infection (23).  
101 Together, these results suggest that *cagY* recombination serves as an immune sensitive  
102 molecular rheostat that “tunes” the host inflammatory response so as to maintain persistent  
103 infection.

104         Here we examined the mechanism by which recombination in *cagY* alters T4SS  
105 function. Since CagY forms the spokes of a T4SS core complex, together with CagX, CagM,  
106 CagT and Cag3 (24, 25), one possibility is that changes in the MRR alter T4SS function by  
107 modifying essential protein-protein interactions, or changing the pore through which effectors  
108 must travel. Alternatively, since CagY recombination occurs in the MRR, which is predicted to  
109 extend extracellularly, allelic variation in CagY might alter integrin binding. At first glance this  
110 seemed unlikely since there are multiple *cagPAI* proteins that bind integrins. Surprisingly, our  
111 results demonstrate that indeed recombination in the CagY MRR alters binding to  $\beta_1$  integrin,  
112 which in turn modulates T4SS function. Moreover, the CagY MRR is expressed on the bacterial  
113 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.

116

## RESULTS

### 117 ***H. pylori* binds to $\alpha_5\beta_1$ integrin in a host cell-free assay**

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

134 Fluorescently stained *H. pylori* was also readily visualized adherent to  $\alpha_5\beta_1$  integrin (Fig  
135 1D). *H. pylori* strains J166 and PMSS1 both attached to  $\alpha_5\beta_1$  integrin in a concentration-  
136 dependent manner and reached saturation at an optical density (OD<sub>600</sub>) of 0.8 (Fig 2A). This  
137 correlates with approximately  $4 \times 10^8$  bacterial cells per ml and was used for all subsequent  
138 experiments. Binding was blocked by pre-incubating the integrin-coated cover slips with P5D2  
139 anti- $\beta_1$  antibody, which sterically inhibits integrin-dependent binding (Fig 2B). Allosterically  
140 stabilizing the  $\alpha_5\beta_1$  integrin in the low affinity conformation by pre-incubation with SG19 antibody  
141 decreased *H. pylori*-integrin binding, while the TS2/16 antibody that locks  $\alpha_5\beta_1$  in the high affinity



142 conformation yielded binding similar to that of an isotype control, indicating that the majority of  
143 derivatized  $\alpha_5\beta_1$  is active (Fig 2B). This was also supported by the observation that pretreatment  
144 of the microfluidic channels with  $Mn^{2+}$  to lock  $\alpha_5\beta_1$  integrin in the high affinity state yielded *H.*  
145 *pylori* adherence similar to that with the TS2/16 antibody and the isotype control (Fig 2B).  
146 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.*  
147 *pylori* binds specifically and in a conformation dependent manner to  $\alpha_5\beta_1$  integrin.

148

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

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

168

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  $\Delta cagPAI$ , 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 J166 $\Delta cagPAI$ . The same results were found for *H. pylori* PMSS1 WT and  $\Delta cagPAI$  (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  $\Delta cagY$ . 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 *cagPAI* 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  $\Delta cagI/L$  and  $\Delta cagE$ , which do  
193 not form a T4SS pilus (27), is similar to WT (Fig 2D).

194

## 195 **The CagY MRR is necessary for integrin binding**

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

211

## 212 **Variation in the motif structure of the CagY MRR alters binding to $\alpha_5\beta_1$ integrin and T4SS** 213 **function.**

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

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

236

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

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

255

## DISCUSSION

256 *H. pylori* persistence in the gastric mucosa is often attributed to evasion of the innate  
257 and adaptive immune response, including antimicrobial peptides (32), toll like receptor signaling  
258 (33, 34), and T cell proliferation (35, 36), as well as promotion of a regulatory T cell response  
259 (37). However, the very presence in most strains of the *cagPAI*, which promotes the host  
260 immune response (38, 39), and the uniform occurrence of gastritis in infected patients, suggest  
261 the possibility that the host inflammatory response may at the same time actually promote *H.*  
262 *pylori* colonization, a concept that has recently been elegantly demonstrated for several enteric  
263 pathogens (40). This is supported by observations of functional antagonism between some *H.*  
264 *pylori* virulence factors such as the CagA oncoprotein and the VacA cytotoxin (41, 42), and by  
265 recent evidence that CagA-dependent inflammation may be important for acquisition of  
266 essential nutrients such as iron (43, 44) and zinc (45). This more nuanced view of the  
267 relationship between *H. pylori* and the host immune response suggests that the overarching  
268 strategy used by *H. pylori* to persist in the stomach might be better characterized as immune  
269 regulation rather than simply immune evasion.

270 CagY is an essential component of the *H. pylori* T4SS that may be well-suited to serve  
271 this immune regulatory function. The *cagY* gene has in its middle repeat region (MRR) a series  
272 of direct DNA repeats that *in silico* predict in-frame recombination events. Recombination in the  
273 *cagY* MRR is in fact common, since variants can be readily detected *in vitro*, though it remains  
274 possible that the frequency is increased in response to unknown host signals. We previously  
275 showed that *cagY* recombination *in vivo* yields a library of insertions and deletions in the MRR,  
276 which maintain CagY expression but frequently alter T4SS function (21). CagY-dependent  
277 modulation of T4SS function is graded—more like a rheostat than a switch—and can yield  
278 variants that confer both gain and loss of function *in vivo* (21, 23). Adoptive transfer and  
279 knockout mouse experiments demonstrate that development of variant *cagY* alleles requires a  
280 CD4<sup>+</sup> T cell- and IFN $\gamma$ -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 *cagPAI* 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 CagL as a  $\beta_1$  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  $\alpha_5\beta_1$

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

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

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

343

## MATERIALS AND METHODS

344

### 345 **Construction and culture of *E. coli* expressing InvA**

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

356

### 357 ***H. pylori* strains and culture conditions**

358 Wild type *H. pylori* strains were cultured on Brucella agar or in Brucella broth  
359 (BBL/Becton Dickinson, Sparks, MD) supplemented with 5% heat-inactivated newborn calf  
360 serum (Invitrogen, Carlsbad, CA) and antibiotics (trimethoprim, 5 mg/liter; vancomycin, 10  
361 mg/liter; polymyxin B, 2.5 IU/liter, amphotericin B, 2.5 mg/liter). *H. pylori* mutant strains were  
362 cultured as for wild type, but with the addition of kanamycin (25 mg/liter), chloramphenicol (5  
363 mg/liter), or streptomycin (10 mg/liter) as appropriate (all antibiotics from Sigma). *H. pylori* liquid  
364 cultures were grown overnight to an optical density at 600 nm (OD<sub>600</sub>) of approximately 0.3 to  
365 0.4. All *H. pylori* cultures were grown at 37°C under microaerophilic conditions generated by a  
366 fixed 5% O<sub>2</sub> concentration (Anoxomat, Advanced Instruments, Norwood, MA). A complete list of  
367 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 J166 $\Delta$ *cagA*, J166 $\Delta$ *cagI/L*  
371 and J166 $\Delta$ *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<sup>®</sup> 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 *cagPAI* (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* cassette, 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*.

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

400

#### 401 **Microfluidic adhesion assay**

402 Microfluidic adhesion assays were assembled as previously described (57). In brief, 25  
403 mm diameter, #1.5 glass coverslips were piranha etched to remove organic molecules and  
404 treated with 1% 3-aminopropyltriethoxysilane to add aminosilane groups. Recombinant human  
405  $\alpha_4\beta_1$ ,  $\alpha_5\beta_1$  or  $\alpha_L\beta_2$  integrin (R&D Systems, Minneapolis, MN) was adsorbed at 10 mg/liter  
406 concentration overnight at 4°C resulting in approximately 2000 sites/ $\mu\text{m}^2$ . Coverslips were then  
407 washed and blocked with Hank's balanced salt solution with 0.1% human serum albumin.  
408 Where indicated, the blocking solution was supplemented with 5 mg/liter of anti-integrin  $\beta_1$   
409 blocking antibody P5D2 (Abcam, San Francisco, CA), low affinity locking anti-integrin  $\beta_1$   
410 antibody SG19, high affinity locking anti-integrin  $\beta_1$  antibody TS2/16 (both from Biolegend, San  
411 Diego, CA), isotype control antibody B11/6 (Abcam, San Francisco, CA), or 2 mM  $\text{MnCl}_2$  ( $\text{Mn}^{2+}$ )  
412 to activate integrin. Custom multi-channel microfluidic device (57) was vacuum sealed, outlets  
413 were attached to Exigo pumps to provide the negative pressure necessary to induce shear, and  
414 inlet reservoirs were loaded with *E. coli* or *H. pylori*. Prior to loading, liquid cultured bacteria  
415 were stained at an  $\text{OD}_{600}$  of 0.8 with 2% Vybrant Dil, DiD or DiO Cell-Labeling Solution (Grand  
416 Island, NY) in Brucella broth for 20 min at 37°C in the dark. Stained bacteria were washed twice  
417 with PBS and then resuspended in Brucella broth to the desired final  $\text{OD}_{600}$ . Competitive binding

418 assays were performed by mixing differently labeled WT and mutant bacteria at an OD<sub>600</sub> of 0.4  
419 (total OD<sub>600</sub> 0.8). Shear was induced at 1 dyne/cm<sup>2</sup> for 3 minutes followed by a 3 minute period  
420 of no shear incubation to allow attachment. Then shear was increased to 1 dyne/cm<sup>2</sup> and 10  
421 second videos were taken along the centerline of the channel in four field of views using an  
422 inverted TIRF research microscope (Nikon) equipped with a 60X numerical aperture 1.5  
423 immersion TIRF objective and a 120 W arc lamp to capture epi-fluorescence images with the  
424 appropriate filter sets (488 nm for DiO, 510 nm for DiD and 543 nm for Dil). Images were  
425 captured using a 16-bit digital complementary metal oxide semiconductor Zyla camera (Andor,  
426 Belfast BT12 7AL, UK) connected to a PC (Dell) with NIS Elements imaging software (Nikon,  
427 Melville, NY). Images were collected with 2x2 binning at a resolution of 1024 x 1024 at a rate of  
428 2 frames per second. Adherent bacteria were identified by the presence of fluorescence, which  
429 was cross-checked with an overlaid brightfield image to eliminate fluorescent noise. Small  
430 numbers of bacteria that were unstained (typically ~10%) were not counted. Bacteria that  
431 remained stationary or tethered after 10 seconds were counted visually in 3 fields of view, and  
432 the results were averaged for each biological replicate. To assess reliability, two observers (one  
433 blinded) independently scored adherent bacteria at 488 nm and 543 nm in 9 fields of view that  
434 contained competitive binding assays (WT and mutant). Mean similarity for the 18 observations  
435 was 0.94, which was calculated as  $1 - \frac{|O_1 - O_2|}{\frac{1}{2}(O_1 + O_2)}$ , where O<sub>1</sub> and O<sub>2</sub> are the  
436 independent scores for the two observers, and a value of 1.0 indicates perfect agreement. Data  
437 on integrin binding are representative of at least three biological replicates, which in most cases  
438 examined three fields of view in duplicate technical replicates.

439

#### 440 **Sequencing of *cagY***

441 The DNA sequences of *cagY* from *H. pylori* PMSS1 and KUS13A and B were  
442 determined using single molecule real-time sequencing (Pacific Biosciences, Menlo Park, CA).  
443 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<sub>600</sub> 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  $\Delta cagY_{MRR}$ , an in-frame deletion of the MRR, was performed using antiserum

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

479

#### 480 **IL-8 ELISA**

481 IL-8 was measured essentially as described previously (59). Human AGS gastric  
482 adenocarcinoma cells (ATCC, Manassas, VA) were grown in RPMI 1640 supplemented with  
483 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin at 5% CO<sub>2</sub>, 37°C.  
484 All antibiotics were excluded from the growth media 24 h prior to *H. pylori* co-culture.  
485 Approximately 5x10<sup>5</sup> human AGS gastric adenocarcinoma cells were seeded in six well plates  
486 with 1.8 ml RPMI/10% fetal bovine serum, incubated overnight, and then co-cultured with  
487 bacteria diluted in 200 µl Brucella broth to give an MOI of 100:1. Brucella broth with no bacteria  
488 served as a baseline control. Supernatants were harvested after 20-22 hours of culture (37°C,  
489 5% CO<sub>2</sub>), stored at -80°C, and then diluted 1:8 prior to IL-8 assay by ELISA (Invitrogen,  
490 Camarillo, CA) performed according to the manufacturer's protocol. WT *H. pylori* J166 or  
491 PMSS1 and its isogenic *cagY* deletion were included on every plate as positive and negative  
492 controls, respectively. IL-8 values were normalized to WT *H. pylori* determined concurrently.

493

#### 494 **High resolution field-emission gun scanning electron microscopy analyses**

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

504

#### 505 **Statistical analysis**

506 Data are reported as mean  $\pm$  SEM. Multiple groups were compared using ANOVA, with  
507 Tukey's or Bonferroni's post hoc test, or with Dunnett's post hoc test when compared only to  
508 WT. Two group comparisons were performed using Student's *t* test. All analyses were carried  
509 out using GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA). A P-value  
510  $<0.05$  was considered statistically significant.



511

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525

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- 733

734

## FIGURE LEGENDS

735 **Fig 1. Microfluidic detection of bacterial adherence to recombinant  $\alpha_5\beta_1$  integrin.** (A)

736 Schematic diagram and photograph of the microfluidic flow cell assembly. (B)

737 Immunofluorescent detection of InvA on the surface of non-permeabilized IPTG-treated *E. coli*

738 containing the pRI253 plasmid with or without *invA*. (C) Attachment to  $\alpha_5\beta_1$  integrin of IPTG-

739 treated *E. coli*. Each strain was used at an OD<sub>600</sub> of 0.8, labeled with DiD or DiO and assayed

740 separately. (D) Micrograph of *H. pylori* J166 labeled with DiO membrane dye, attached to  $\alpha_5\beta_1$

741 integrin in the microfluidic flow cell. Brightfield and fluorescence overlay of the field of view

742 (FOV) demonstrates fluorescent labeling of *H. pylori*.

743

744 **Fig 2.  $\alpha_5\beta_1$  integrin adherence of WT *H. pylori* J166 and *cagPAI* deletion mutants.** (A)

745 Adherent *H. pylori* J166 and PMSS1 per FOV as a function of bacterial optical cell density at

746 600 nm (OD<sub>600</sub>). (B) Adherent *H. pylori* after pre-incubation of flow cells with B11/6 isotype

747 control antibody, P5D2 antibody to sterically inhibit  $\beta_1$  integrin binding, or antibodies to lock the

748 integrin in the low (SG19) or high (TS2/16) affinity conformation, respectively. Treatment of

749 integrin with Mn<sup>2+</sup> to stabilize the high affinity state produced results similar to treatment with

750 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.

751 (D) Adherence to  $\alpha_5\beta_1$  integrin of J166 WT and deletion mutants, which were fluorescently

752 labeled with DiO and DiI, respectively, mixed in a 1:1 ratio, and enumerated by counting

753 fluorescent bacteria per field of view (FOV). Results are expressed as the ratio of deletion

754 mutant to WT. (E) Steric inhibition with P5D2 antibody (white bars) demonstrated that

755 adherence is integrin-specific.  $\Delta cagY$  adherence was similar with and without steric inhibition,

756 suggesting that it represents only non-specific background binding. Results are mean ( $\pm$  SEM)

757 of 3 to 5 independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

758

759 **Fig 3. Schematic diagram of the *H. pylori* J166 *cagPAI* in the WT and selected deletion**  
760 **mutants.** In J166 $\Delta$ *cagY*, the entire *cagY* gene is replaced by a *cat-rpsL* cassette (streptomycin  
761 susceptibility and chloramphenicol resistance).  $\Delta$ *cagY*<sub>MRR</sub> 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 *cagPAI* 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  $\Delta$ *cagPAI* cultured with or without AGS cells. Pili are indicated by white arrows. Scale  
771 bar, 1  $\mu$ m. (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 ( $\pm$  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  $\Delta$ *cagY* deletion mutants. The corresponding amino acid structure of the

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

796

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

803

## SUPPORTING FIGURE LEGENDS

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

810

811 **S2 Fig. Fluorophore labeling control experiments.** (A) Percent of cells stained with Dil or DiO  
812 ([fluorescently stained cells divided by total cells seen on brightfield] x 100) was determined for  
813 *H. pylori* J166 WT,  $\Delta$ cagPAI,  $\Delta$ cagY, and isogenic strains expressing functional ( $\Delta$ cagY[Out1])  
814 or non-functional ( $\Delta$ cagY[Out3] cagY alleles. (B) *H. pylori* J166 WT and  $\Delta$ cagPAI (each at  
815 OD<sub>600</sub>=0.4) were labeled with Dil or DiO, respectively (left), mixed 1:1, and imaged with a 546  
816 nm (Dil) or 488 nm (DiO) light source. Parallel experiments were performed with a dye swap  
817 (middle). WT labeled with Dil was also mixed with WT labeled with DiO (right). Binding to  $\alpha_5\beta_1$   
818 integrin was similar whether detected with DiO or Dil fluorescent dyes, and whether in  
819 competition with a strain with low or high binding ability. Results for WT measured in a  
820 competition setting were also very similar to that when measured individually (compare with Fig  
821 2B). Data represent mean  $\pm$  SEM of  $\geq 3$  experiments.

822

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

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

833

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

837

838 **S5 Fig. T4SS function in various *cagPAI* *H. pylori* J166 mutants.**

839 IL-8 induction in AGS cell co-cultured with J166 WT,  $\Delta$ *cagY*, *cagXY*, *cagY* and  $\Delta$ *cagY*<sub>MRR</sub>.  
840 \*\*\*P<0.001.

841

842 **S1 Table. Primers used for PCR.**

843

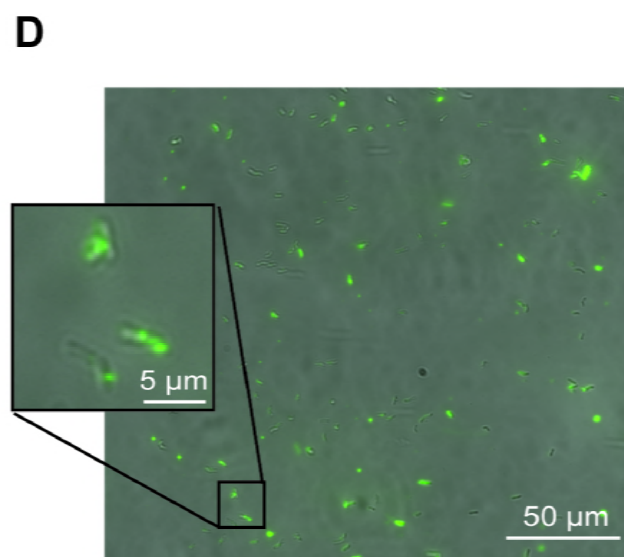
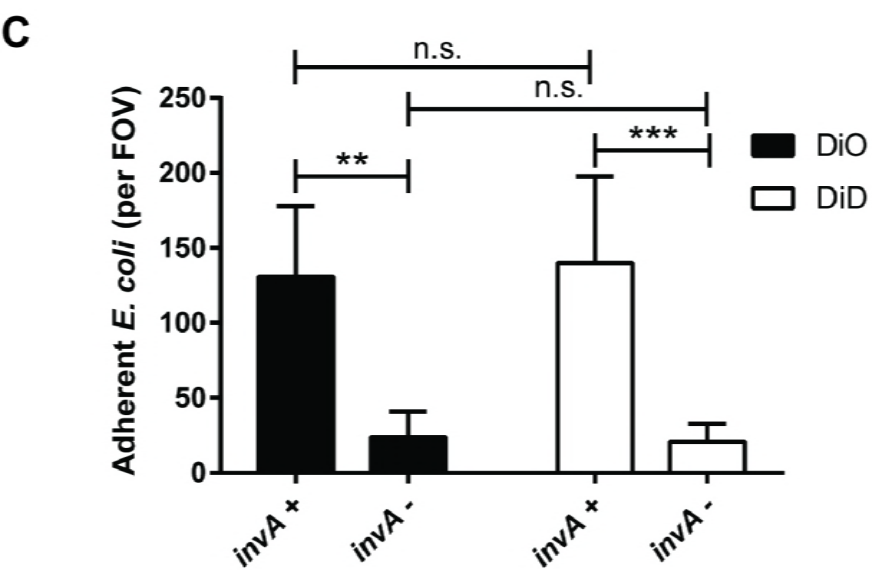
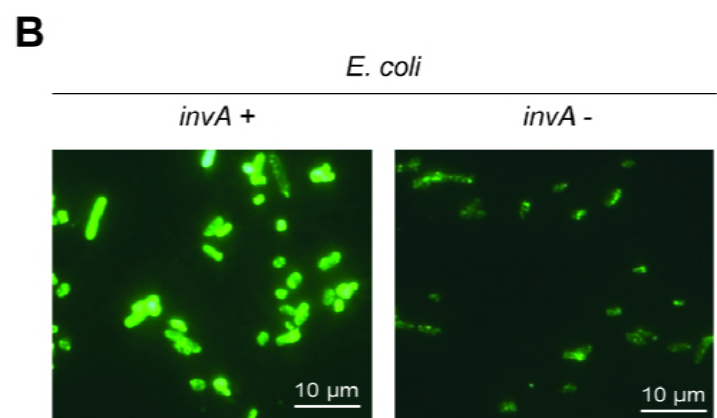
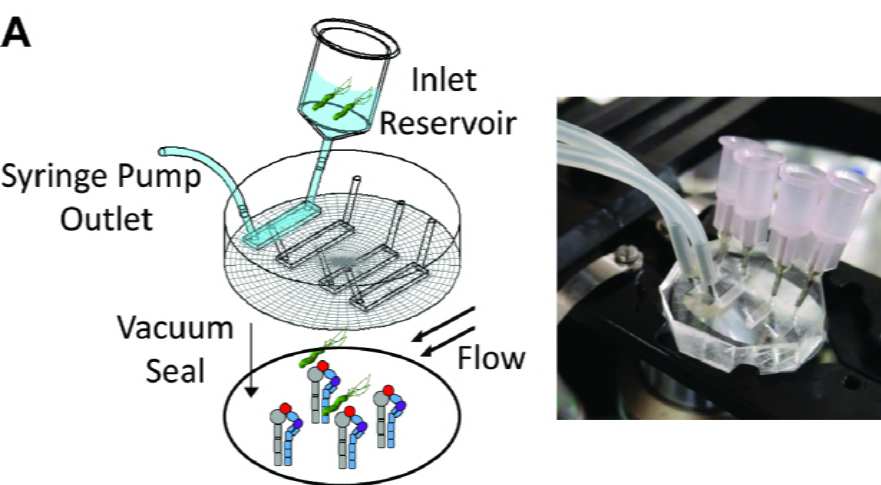
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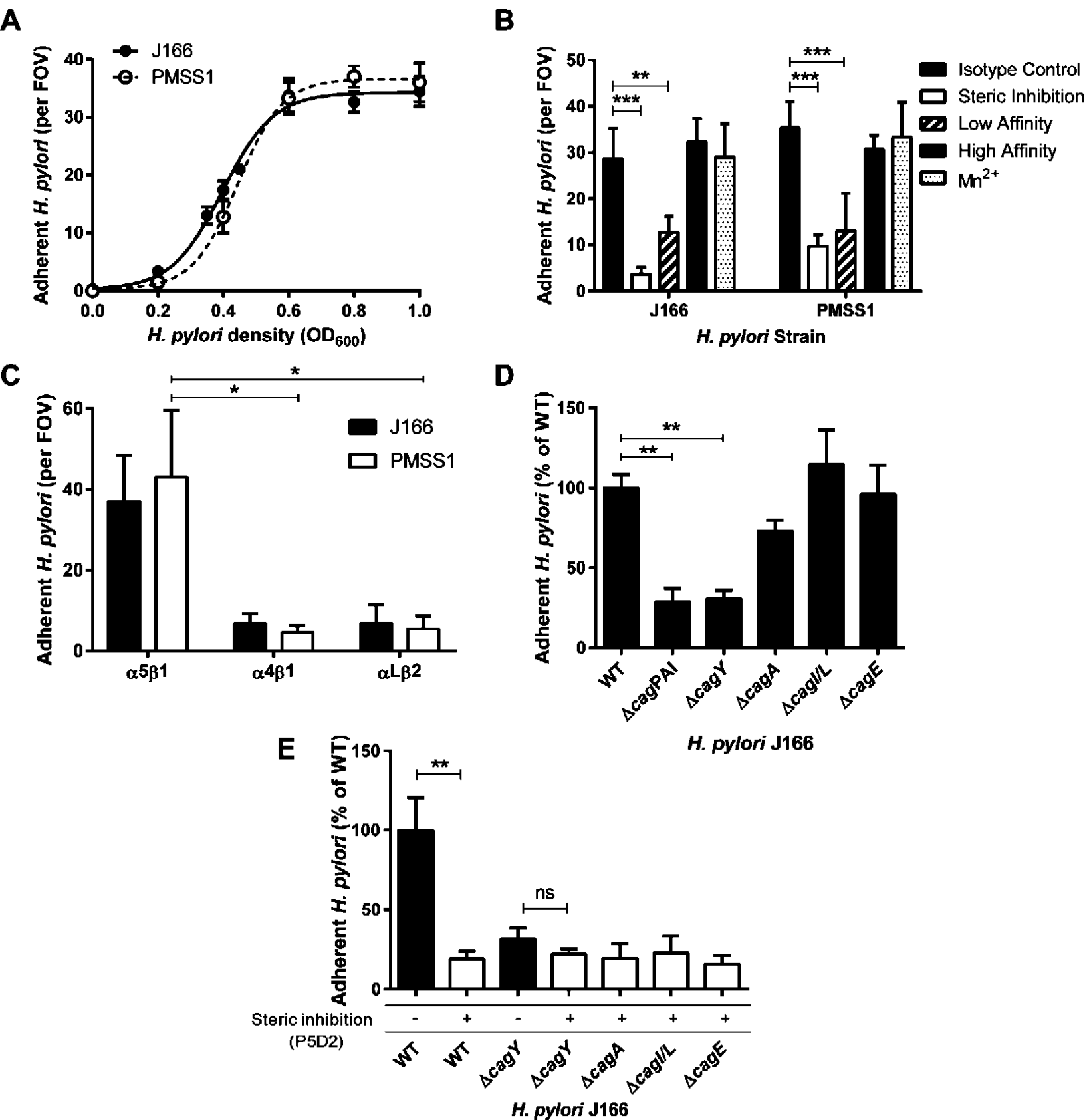


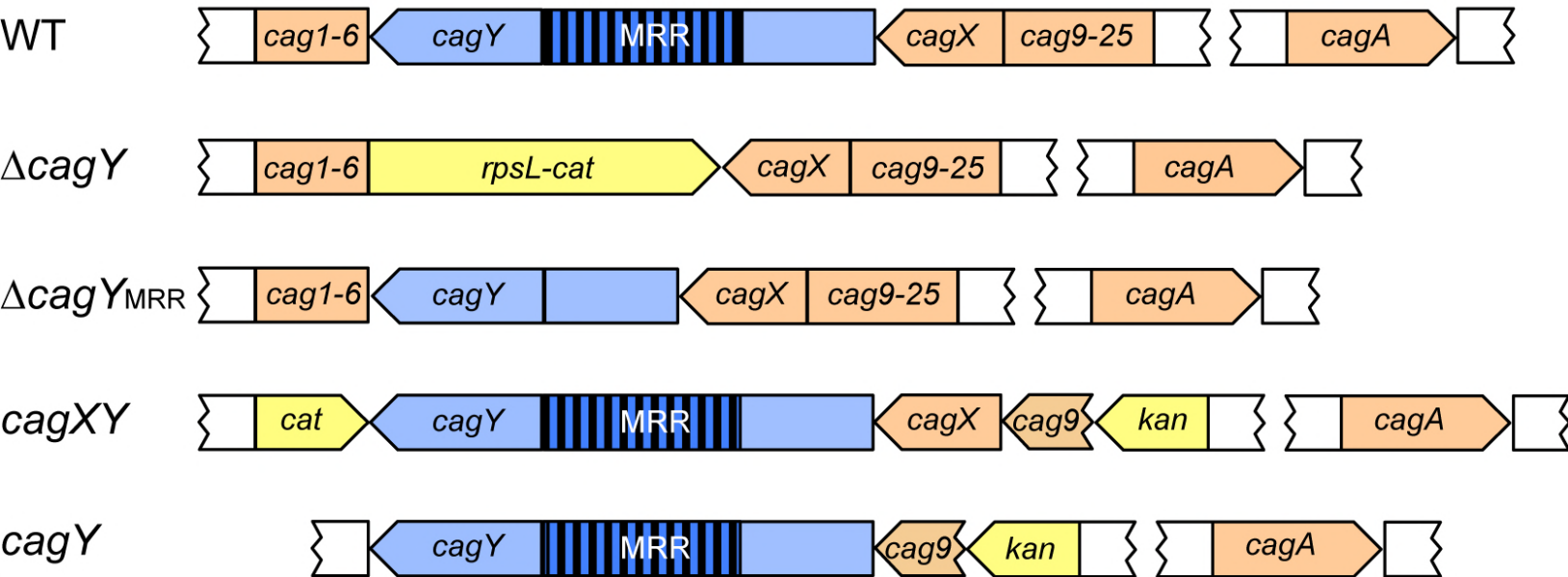
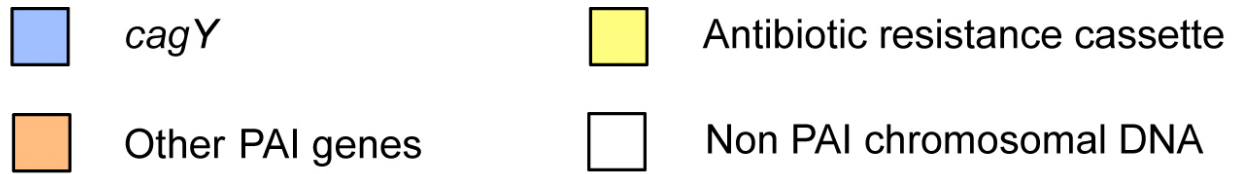
**Table 1. *H. pylori* strains**

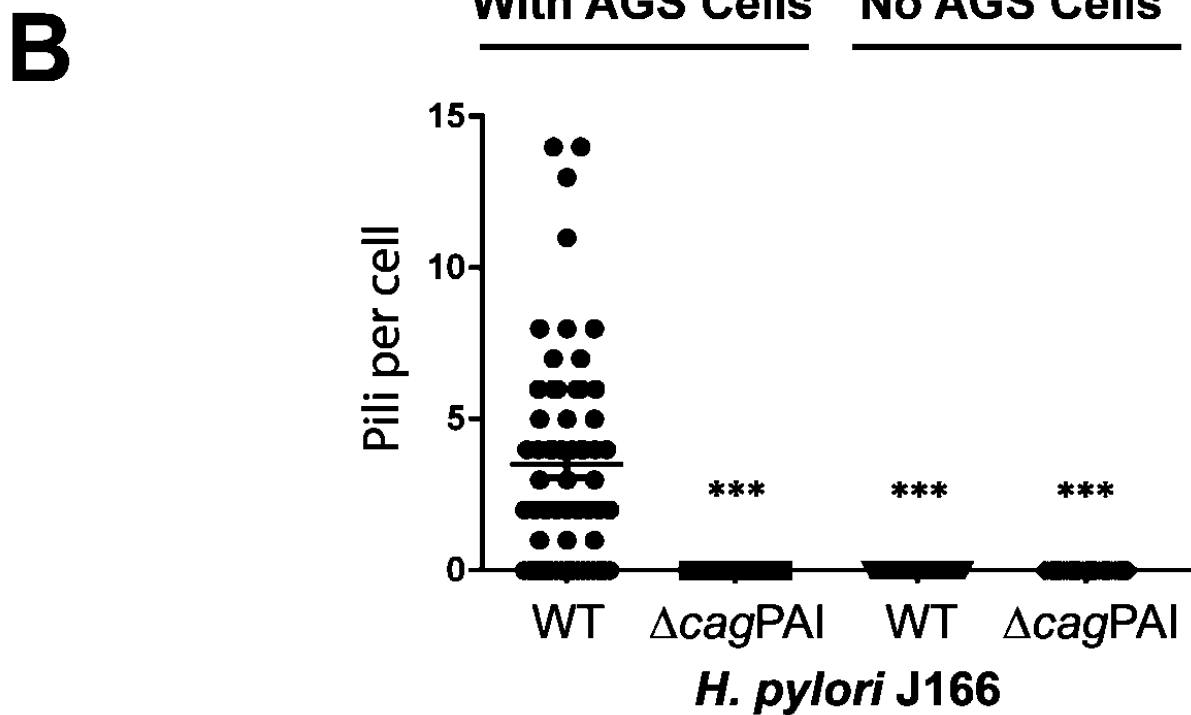
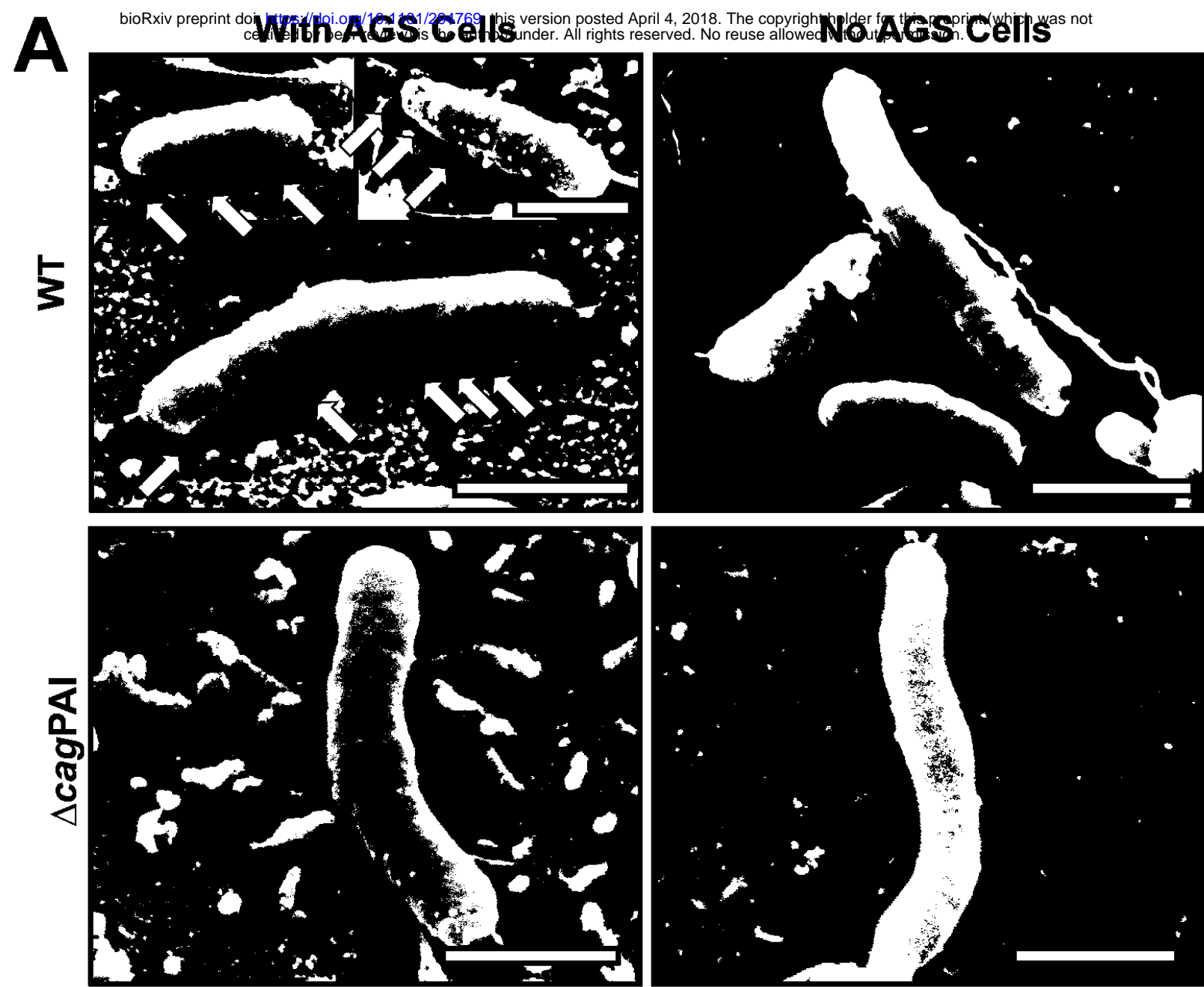
Strain	Relevant characteristic(s)	Antibiotic resistance <sup>a</sup>	Source (Reference)
<i>E. coli</i> strains			
BL21 <i>invA</i> +	<i>E. coli</i> BL21 with plasmid pRI253	Amp	(55)
BL21 <i>invA</i> +	<i>E. coli</i> BL21 with plasmid pRI253Δ <i>invA</i>	Amp	this study
<i>H. pylori</i> wild type strains			
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 <i>cagPAI</i>	Deletion of the entire <i>cagPAI</i>	Cm, Km	(39)
J166Δ <i>cagA</i>	J166Δ <i>cagA</i> :: <i>aphA</i>	Km	this study
J166Δ <i>cagI/L</i>	J166Δ <i>cagI/L</i> :: <i>aphA</i>	Km	this study
J166Δ <i>cagY</i>	J166 Str <sup>R</sup> Δ <i>cagY</i> :: <i>cat_rpsL</i>	Cm	(21)
J166Δ <i>cagE</i>	J166Δ <i>cagE</i> :: <i>aphA</i>	Km	this study
J166 <i>cagXY</i>	J166Δ <i>cag1-6</i> :: <i>cat</i> ,Δ <i>cag9-25</i> :: <i>aphA</i>	Cm, Km	this study
J166 <i>cagY</i>	J166Δ <i>cag1-6</i> , Δ <i>cag8</i> , Δ <i>cag9-25</i> :: <i>aphA</i>	Km	this study
J166Δ <i>cagY</i> <sub>MRR</sub>	J166 Str <sup>R</sup> Δ <i>cagY</i> <sub>MRR</sub>	Str	this study
J166 <i>cagY</i> replacements			
Δ <i>cagY</i> [mOut1]	J166Δ <i>cagY</i> replaced with <i>cagY</i> from mOut1	Str	(21)
Δ <i>cagY</i> [mOut2]	J166Δ <i>cagY</i> replaced with <i>cagY</i> from mOut2	Str	(21)
Δ <i>cagY</i> [mOut3]	J166Δ <i>cagY</i> replaced with <i>cagY</i> from mOut3	Str	(21)
Δ <i>cagY</i> [mOut4]	J166Δ <i>cagY</i> replaced with <i>cagY</i> from mOut4	Str	(21)
PMSS1 <i>cagY</i> replacements			
Δ <i>cagY</i> [Out1]	PMSS1Δ <i>cagY</i> replaced with <i>cagY</i> from Out1	Str	(23)
Δ <i>cagY</i> [Out2]	PMSS1Δ <i>cagY</i> replaced with <i>cagY</i> from Out2	Str	(23)
Δ <i>cagY</i> [Out3]	PMSS1Δ <i>cagY</i> replaced with <i>cagY</i> from Out3	Str	(23)
CagY replacement clinical isolates			
KUS13AΔ <i>cagY</i>	KUS13AΔ <i>cagY</i> :: <i>cat_rpsL</i>	Cm	(23)
KUS13BΔ <i>cagY</i>	KUS13BΔ <i>cagY</i> :: <i>cat_rpsL</i>	Cm	(23)
KUS13AΔ <i>cagY</i> [KUS13B]	KUS13AΔ <i>cagY</i> replaced with <i>cagY</i> from KUS13B	Str	(23)
KUS13BΔ <i>cagY</i> [KUS13A]	KUS13BΔ <i>cagY</i> replaced with <i>cagY</i> from KUS13A	Str	(23)

<sup>a</sup>Amp, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Str, streptomycin

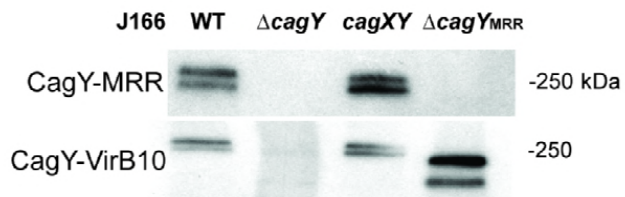








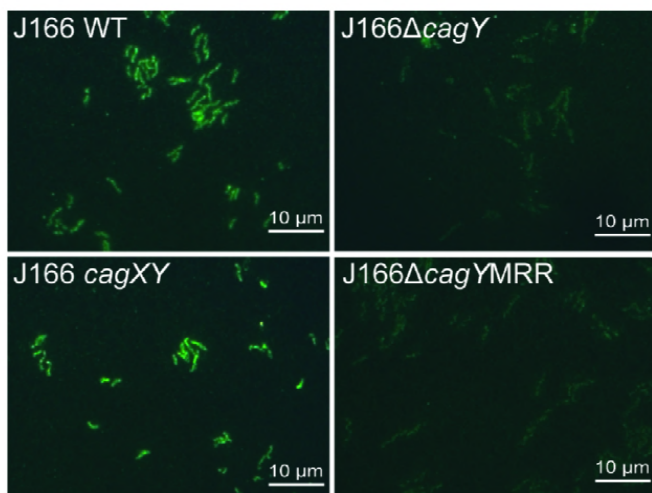
**A**



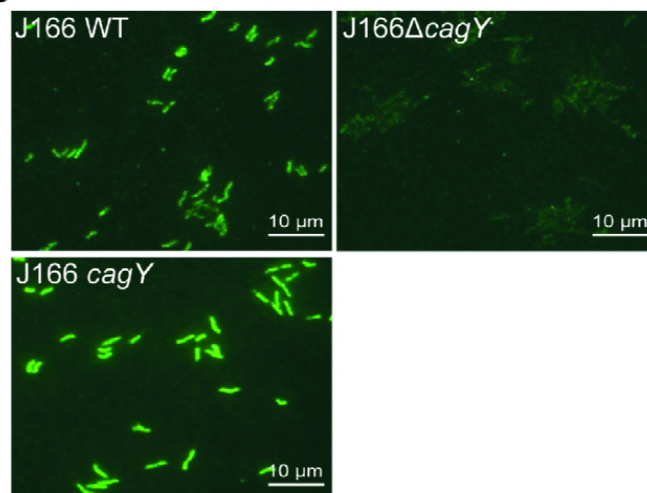
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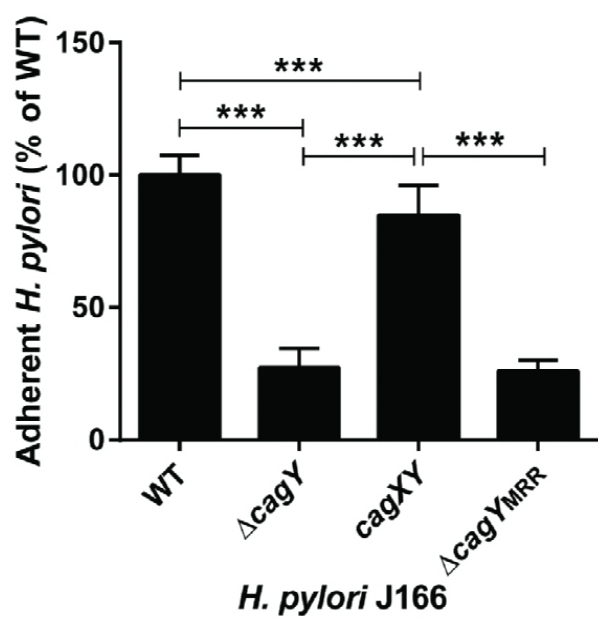
**C**



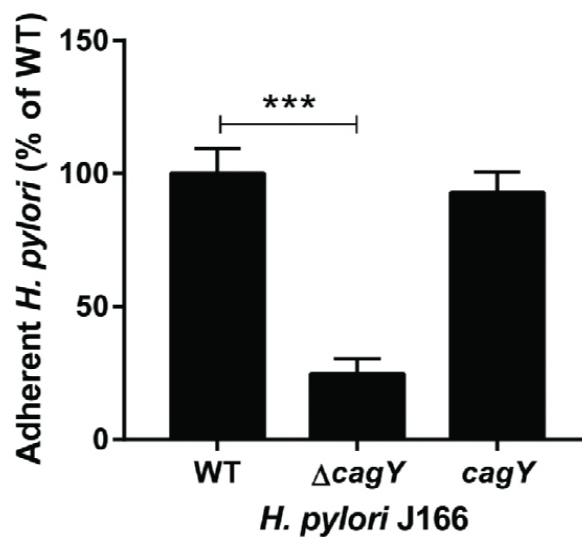
**D**

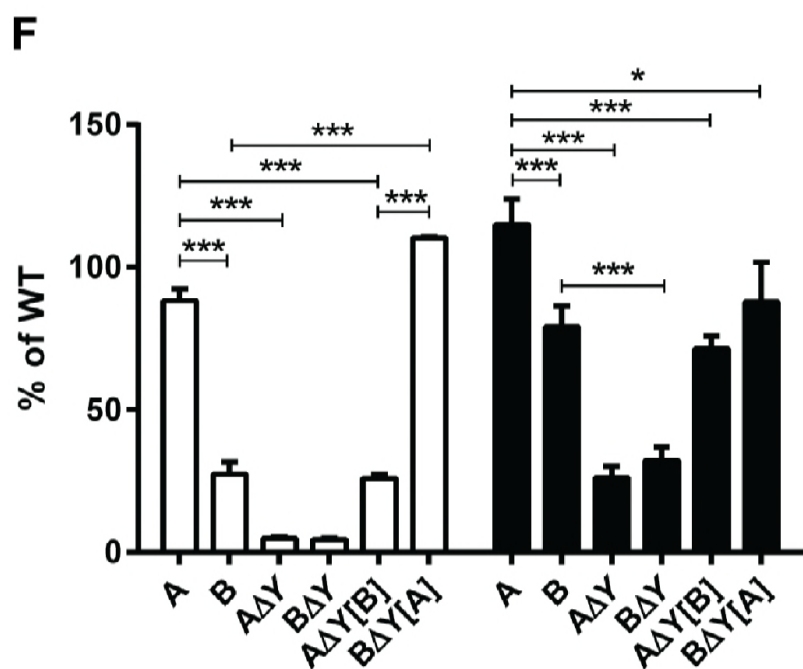
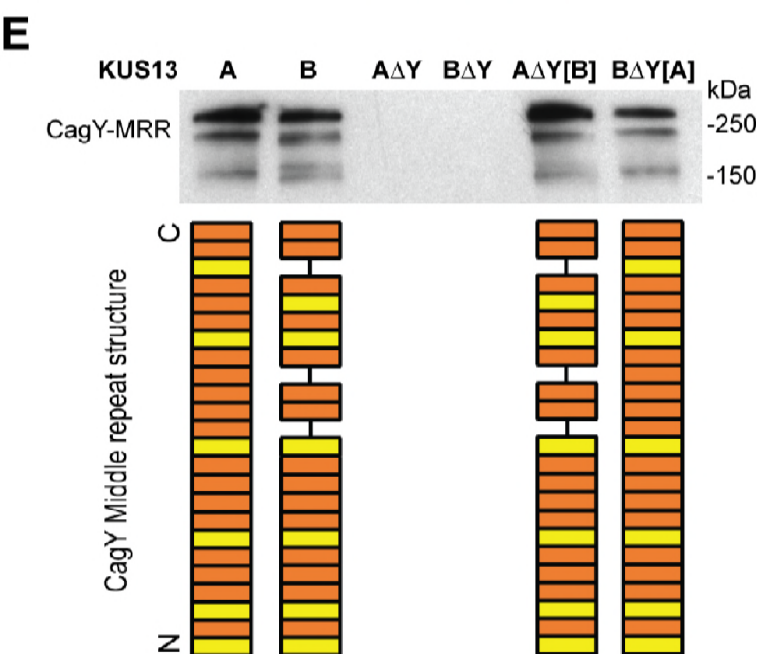
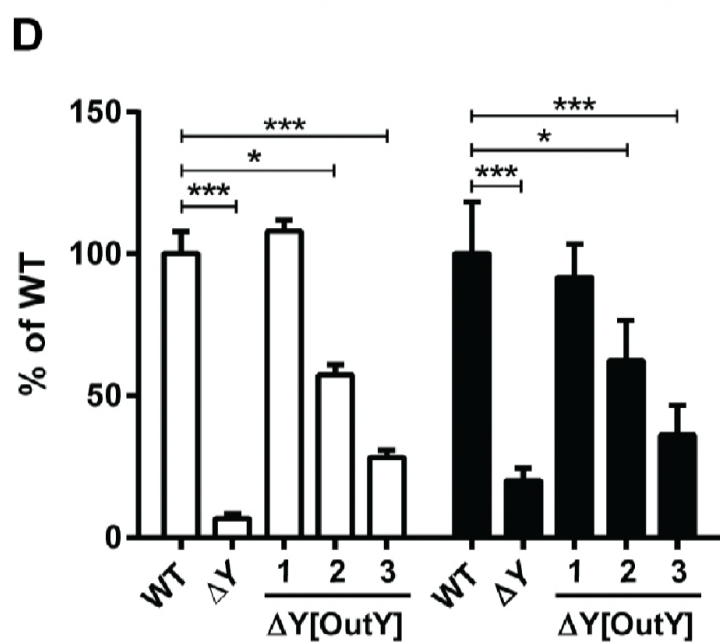
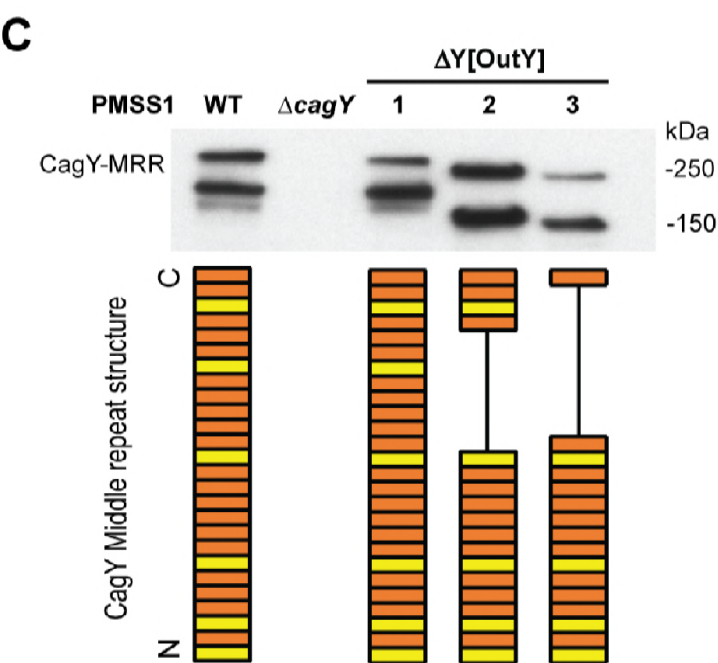
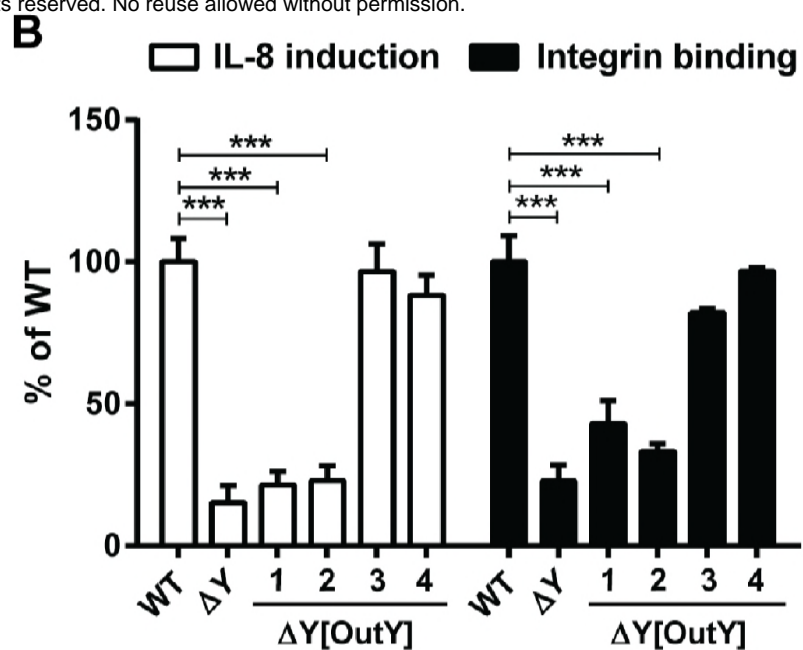
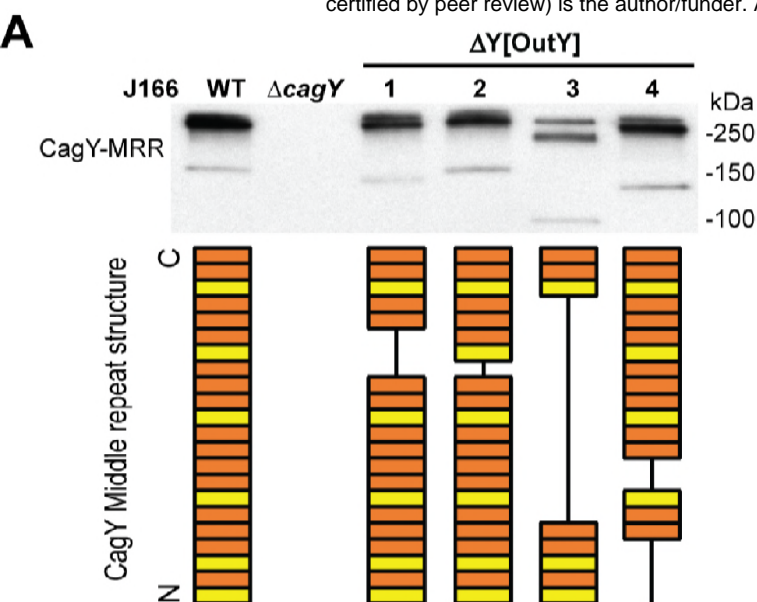


**E**

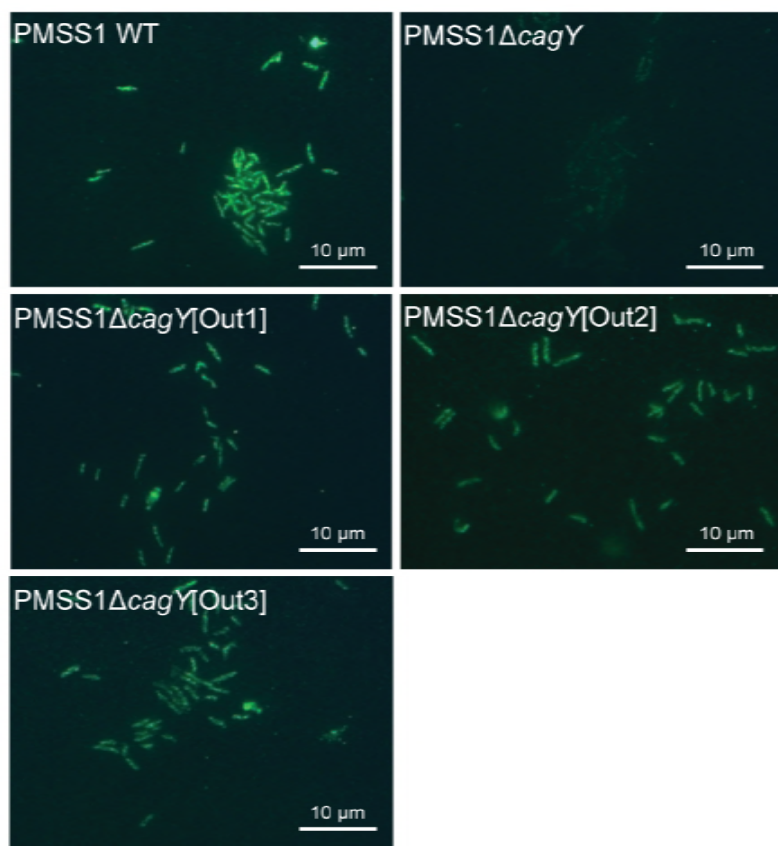


**F**





**A**



**B**

