HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2016 November 10.

Published in final edited form as:

Oncogene. 2016 December 1; 35(48): 6262–6269. doi:10.1038/onc.2016.158.

Pathogenic *Helicobacter pylori* Strains Translocate DNA and Activate TLR9 via the Cancer-Associated *cag* Type IV Secretion System

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Abstract

Helicobacter pylori is the strongest identified risk factor for gastric cancer, the third most common cause of cancer-related death worldwide. An *H. pylori* constituent that augments cancer risk is the strain-specific *cag* pathogenicity island, which encodes a type IV secretion system (T4SS) that translocates a pro-inflammatory and oncogenic protein, CagA, into epithelial cells. However, the majority of persons colonized with $CagA^+$ *H. pylori* strains do not develop cancer, suggesting that other microbial effectors also play a role in carcinogenesis. Toll-like receptor 9 (TLR9) is an endosome bound, innate immune receptor that detects and responds to hypo-methylated CpG DNA motifs that are most commonly found in microbial genomes. High expression *tlr9* polymorphisms have been linked to the development of premalignant lesions in the stomach. We now demonstrate that levels of *H. pylori*-mediated TLR9 activation and expression are directly related to gastric cancer risk in human populations. Mechanistically, we show for the first time that the *H. pylori* cancer-associated *cag* T4SS is required for TLR9 activation and that *H. pylori* DNA

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Supplementary Information accompanies the paper on the *Oncogene* website (http://www.nature.com/onc)

is actively translocated by the *cag* T4SS to engage this host receptor. Activation of TLR9 occurs through a contact-dependent mechanism between pathogen and host, and involves transfer of microbial DNA that is both protected as well as exposed during transport. These results indicate that TLR9 activation via the *cag* island may modify the risk for malignancy within the context of *H. pylori* infection and provide an important framework for future studies investigating the microbial-epithelial interface in gastric carcinogenesis.

Introduction

Helicobacter pylori is a bacterial carcinogen that incurs the highest known risk for gastric cancer (1). Approximately 80% of the gastric cancer burden and 5.5% of all malignancies worldwide are attributable to *H. pylori*-induced injury (1, 2). However, only a subset of chronically colonized individuals ever develop neoplasia. Enhanced risk for gastric carcinogenesis is related to *H. pylori* strain differences, inflammatory responses governed by host genetic diversity, and/or specific interactions between host and microbial determinants (3).

One cancer-linked *H. pylori* locus is the *cag* pathogenicity island, which encodes a type IV secretion system (T4SS) that forms a syringe-like structure to translocate the effector oncoprotein CagA and peptidoglycan into host cells (4–19). T4SSs are common among Gram-negative bacteria due to their versatility in terms of the type and destination of secreted substrates. However, despite the ubiquity of T4SS in Gram-negative bacteria, their ability to mediate trans-kingdom DNA transfer is rare. Indeed, only a very few bacterial T4SSs have been shown to facilitate DNA transfer into yeast (20, 21) or mammalian cells (22–24). The T4SS of *Agrobacterium tumefaciens* is the only known example of a pathogenesis-associated T4SS that facilitates plasmid DNA transfer into a eukaryotic host (25–27). In this system, *A. tumefaciens* translocates the tumor-inducing Ti-plasmid into plant cells, which is then incorporated into the genome and ultimately results in malignant transformation. *A. tumefaciens* T4SS-mediated DNA transfer has also been shown to promote transformation of human cells under non-physiological conditions (28). Of particular interest, the *A. tumefaciens* archetypal T4SS retains a high level of homology to the *cag* type IV secretion system, suggesting that *H. pylori* has the ability to translocate DNA into host cells via the *cag* island.

In addition to the *cag* T4SS, host factors have also been implicated in augmenting *H. pylori*-induced gastric cancer risk. Toll-like receptors (TLRs) orchestrate host immune responses targeting pathogens via selective recognition of pathogen-associated molecular patterns (PAMPs) (29); however, chronic activation of TLRs in the gastric niche has been implicated in promoting carcinogenesis (30). TLR9 is an intracellular receptor that recognizes hypo-methylated CpG motifs (31), which are abundant in DNA of bacterial, viral, or synthetic origin, but are atypical within mammalian genomes (32). TLR9 expression is up-regulated in human gastric cancer specimens, and *H. pylori* DNA has been shown to directly promote cancer cell invasion (33–38). Moreover, polymorphisms in the *tlr9* gene have also been shown to increase the risk for development of both pre-malignant and malignant gastric lesions (30, 39). Therefore, we utilized human gastric specimens and *in vitro* models of

microbial-epithelial interactions to define the role of *H. pylori* DNA translocation and TLR9 activation in gastric carcinogenesis.

Results and Discussion

In many areas of the world, geographic differences in gastric cancer rates are present despite similarly high *H. pylori* prevalence rates (40). This has been well described in Colombia, where residents of the Andean Mountain region have an extraordinarily high incidence of gastric cancer (150/100 000), compared to inhabitants of the Pacific coast region (6/100 000), despite the fact that greater than 90% of the collective population is infected with *H. pylori* (41, 42). This disparity in gastric cancer risk but not *H. pylori* prevalence (15, 43–45) provides a unique opportunity to define microbial and host determinants that play a role in gastric carcinogenesis. Therefore, we first analyzed *H. pylori* strains isolated from the high and low gastric cancer risk regions for their ability to activate TLR9 using a specific HEK293-hTLR9 reporter assay system. In this reporter assay, HEK293 cells, which are inherently devoid of most innate immune receptors, are stably transfected with either a TLR9 expression plasmid and an NFκB/AP-1-linked SEAP reporter (TLR9+), or a control NFκB/AP-1 SEAP reporter alone (Parental). Cells were challenged with *H. pylori* strains isolated from patients residing in either the high-risk or low-risk region. *H. pylori* strains isolated from the high-risk region induced significantly higher levels of TLR9 activation compared to strains harvested from patients in the low-risk region (Figure 1a). To determine whether the ability of high-risk *H. pylori* isolates to activate TLR9 *in vitro* translated into the cognate gastric niche, levels of epithelial TLR9 expression in gastric biopsies obtained from infected patients were quantified. Gastric epithelial TLR9 expression levels were significantly increased in patients residing in the high-risk region compared to the low-risk region (Figure 1b,c), findings that mirror the *in vitro* data. Patients residing in the high cancer-risk region also displayed significantly increased histological scores compared to patients in the low cancer-risk region (mean ± SEM; 3.89±0.26 vs. 2.05±0.11, respectively; $p \leq 0.0001$). Additionally we observed that TLR9 expression was more extensive and involved more regions of the gastric glands in patients residing in the high-risk region, frequently extending from the base to the gastric pit. Collectively, these results indicate that *H. pylori* strains linked to an increased risk for gastric cancer induce more intense TLR9 activation *in vitro* and enhanced TLR9 expression *in vivo*.

Despite the evolutionary homology of the *cag* T4SS to the archetypal *A. tumefaciens* T4SS (46), DNA translocation by the *H. pylori cag* system has never been demonstrated. Therefore, to define mechanisms through which *H. pylori* activates TLR9, we subsequently conducted experiments using the well-characterized wild-type and mutant *H. pylori* cag^+ strains J166, 26695, and 7.13 (12, 47, 48). Previous reports have shown that *H. pylori* DNA induces TLR9 activation in immune cells (49–51); thus, we first confirmed that purified *H. pylori* genomic DNA (gDNA) could induce TLR9 activation in our HEK293 reporter assay (Figure 2a). We also demonstrated that viable wild-type cag^+ *H. pylori* could activate TLR9 to significantly increased levels compared to controls (Figure 2b, 2c, S1). The specificity of the TLR9-dependent response was then validated in HEK293-hTLR9 reporter cells that were pre-treated with the endosomal inhibitor chloroquine, which blocks TLR9 activation.

H. pylori-infected reporter cells pre-challenged with chloroquine displayed a significant decrease in TLR9 activation compared to control cells (Figure S2).

H. pylori possesses up to four potential T4SSs with the putative ability to translocate DNA to and from the bacterial cell: the *comB* DNA uptake (competence) system, the *cag* system, as well as the *tfs3* and *tfs4* secretion systems (52–54). Analysis of an *H. pylori comB* DNA uptake mutant revealed no differences in levels of TLR9 activation compared to the *H. pylori* wild-type strain J166 (Figure 2b). Therefore, we next investigated the role of the *cag* T4SS in mediating TLR9 activation. TLR9 reporter cells were challenged with either an isogenic *cagA*$^-$ mutant (which lacks the effector protein CagA), *cagE*$^-$ or *cagY*$^-$ mutants (which encode essential proteins for T4SS assembly), or a *pgdA*$^-$ mutant (which reduces peptidoglycan-mediated NOD1 activation (55)). Loss of *cagA* or *pgdA* had no effect on TLR9 activation; however, *cagE*$^-$ or *cagY*$^-$ mutants were incapable of activating TLR9, suggesting that DNA translocation to the host cell and subsequent TLR9 activation may occur via the *cag* T4SS (Figure 2b). The requirement for a functional *cag* T4SS to induce TLR9-dependent responses was also demonstrated in two independent *H. pylori cag*$^+$ strains, 26695 and 7.13, as mutations in *cag* T4SS structural components abrogated TLR9 activation (Figure 2c, S1). Importantly, *H. pylori* strain 26695, which lacks intact *tfs3* and *tfs4* gene clusters (53), still induced robust TLR9 activation (Figure 2c). We also observed TLR9 activation following infection with *H. pylori* strains devoid of plasmid DNA (strain J166, 26695, Figure 2b, 2c (12, 47)), suggesting that other forms of DNA can be translocated by the *cag* T4SS. This observation is of particular importance because it highlights fundamental differences between *H. pylori* DNA translocation and DNA translocation that occurs in other bacterial species, which only translocate plasmid DNA into host cells. To extend these findings, we also infected AGS gastric epithelial cells with wild-type *H. pylori cag*$^+$ strains (J166 or 26695), their respective *cagE*$^-$ mutants, or their purified genomic DNA and assessed the levels of IL-8 secretion as a potential downstream target of TLR9 mediated NF-κB activation (Figure 2d). We found that the *cagE*$^-$ mutants induced significantly decreased levels of IL-8 compared to wild-type *H. pylori* strains. Additionally, purified gDNA from the wild-type strains induced a significantly increased level of IL-8 production compared to vehicle control, suggesting that *H. pylori* gDNA can not only activate TLR9, but can also induce production of a known downstream target of this receptor. Collectively, these data indicate that a functional *H. pylori* cancer-linked T4SS is required for TLR9 activation, but the known effector molecules translocated by this system (CagA and peptidoglycan) are dispensable for this phenotype.

H. pylori is predominantly an extracellular pathogen and TLR9 is an endosomal receptor, implying that DNA is either directly injected into the host cell, or is taken up via a different mechanism that is enhanced by a functional T4SS. To determine whether TLR9 was activated by *H. pylori* DNA entering host cells via a host-induced pathway, TLR9 reporter cells were incubated with bacterial cell-free, *H. pylori* conditioned media at concentrations up to 30%; however, no changes in TLR9 activation were observed compared to control cells (Figure 2e). These data indicate that activating DNA is not secreted, in contrast to strategies employed by *Neisseria* in which DNA is translocated into the extracellular milieu (56). Although primarily extracellular, *H. pylori* does retain the capacity to invade host epithelial cells in a limited fashion (55). Therefore, we next quantified intracellular *H. pylori* viability

within HEK293-hTLR9 reporter cells. Wild-type *H. pylori* survived equally well compared to the corresponding *cag* mutants (Figure 2f), despite marked differences in TLR9 activation induced by these strains (Figure 2b). These results indicate that microbial endocytosis does not affect *H. pylori*-induced TLR9 activation since only wild-type *H. pylori* were capable of activating this endosome-bound receptor.

To determine whether the *cag* T4SS is required for direct DNA delivery into host cells, *H. pylori* DNA was labeled with BrdU and bacteria were subsequently co-cultured with AGS gastric epithelial cells. Using this technique, bacterial DNA can be easily distinguished from host DNA via incorporation of BrdU. Structured illumination microscopy (SIM) demonstrated that wild-type *H. pylori* translocated BrdU-labeled DNA into host cells (Figure 3a, Figure S3, Figure S4 movie a,b). However, intracellular BrdU-labeled DNA was not observed in host cells infected with the *H. pylori cagE*$^{-}$ mutant (Figure 3a, Fig S5 movie). To confirm and quantify these results using an independent methodology, host intracellular levels of BrdU-labeled *H. pylori* DNA were assessed via flow cytometry. Levels of intracellular DNA were significantly increased in *H. pylori* wild type-infected compared to uninfected AGS cells. A significant reduction was observed in cells infected with the *cagE*$^{-}$ mutant compared to wild-type infected AGS cells (Figure 3b), albeit not to levels observed in uninfected cells, which may be due to adherent and/or invasive *H. pylori* that could not be completely removed prior to analysis. Collectively, these data demonstrate that *H. pylori* utilizes the *cag* T4SS to translocate microbial DNA into eukaryotic cells, thereby activating TLR9.

Elaboration of the cancer-associated *cag* T4SS develops in response to direct host cell contact, and effector translocation is dependent on assembly of the T4SS apparatus. We therefore performed orthologous mechanistic studies to investigate whether TLR9 activation required *de novo* synthesis of Cag proteins for apparatus biogenesis, or whether *cag* T4SS-dependent DNA transfer could be achieved using pre-assembled protein complexes. Treatment of *H. pylori*-HEK293 co-cultures with bacteriostatic, sub-lethal concentrations of chloramphenicol, an inhibitor of bacterial protein synthesis, revealed a significant reduction in levels of TLR9 activation (Figure 4a). Chloramphenicol-mediated inhibition of *H. pylori*-driven TLR9 activation occurred in a dose-dependent manner but did not affect *H. pylori* viability (Figure 4b). These data indicate that *cag* T4SS-mediated DNA translocation requires new protein synthesis.

DNA transfer by *A. tumefaciens* occurs via substrate delivery both within and on the external surface of the T4SS conduit (25). To further investigate the mechanisms of *cag* T4SS-dependent DNA transfer into host cells in greater depth, we assessed whether exogenous DNase I treatment would compromise the integrity of transported DNA and impair TLR9 activation. Compared to untreated controls, levels of TLR9 activation decreased by approximately 50% when DNase I was added during *H. pylori*-HEK293 cell co-culture (Figure 4c). In parallel, we analyzed the ability of monoclonal anti-DNA antibodies to functionally block *H. pylori*-dependent TLR9 activation. Compared to untreated controls, *H. pylori*-mediated TLR9 activation was significantly reduced in the presence of pan anti-DNA antibodies, an effect that was titratible (Figure 4d). Taken together, these data indicate that *cag* T4SS-dependent DNA translocation occurs through a

contact-dependent mechanism that involves transfer of bacterial DNA that is both protected as well as exposed during transport.

In conclusion, we have demonstrated that patients residing within a high gastric cancer risk region of Colombia express significantly higher levels of TLR9 within gastric epithelial cells compared to patients residing in the low risk region, and that the *H. pylori* strains isolated from these patients concomitantly induce higher levels of TLR9 activation. We further demonstrate that *H. pylori* utilizes the *cag* island, a locus known to augment cancer risk, to translocate bacterial DNA into host cells. Collectively these results define a mechanism that may explain previous epidemiologic data linking TLR9 to the development of gastric cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the following core laboratories and personnel at Vanderbilt University for their contributions to these studies: Tissue Acquisition and Pathology Core; Division of Animal Care; Cell Imaging Shared Resource Core (CISR) and the Digestive Disease Research Center. This work was supported by NIH R01-DK58587, R01-CA77955, P01-CA116087, and P30-DK058404 (R.M. Peek Jr.); Vanderbilt CTSA grant UL1 TR000445 from NCATS/NIH (VICTR award VR7227), APS 1-04-520-9211 and institutional funds (M. Hadjifrangiskou); R01-DK053620, R01-CA190612, and a Department of Veterans Affairs Merit Review grant I01BX001453 (K.T. Wilson); and P01-CA028842 (K.T. Wilson and P. Correa). The authors declare no conflict of interest.

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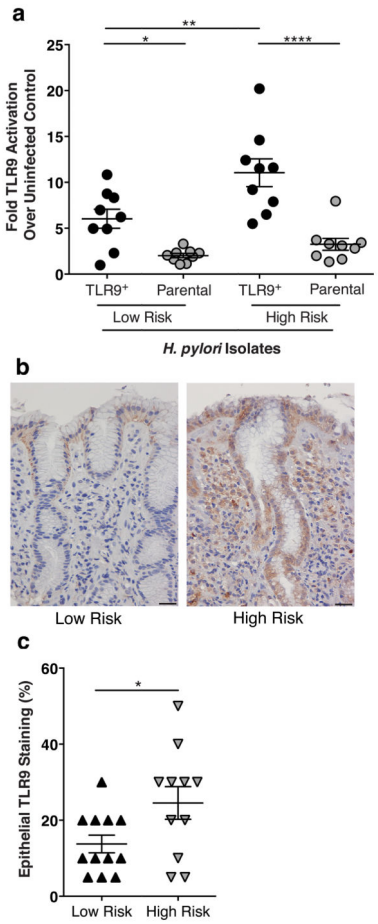

Figure 1. *H. pylori* activation of TLR9 in a human population

(a) TLR9 activation by *H. pylori* isolates obtained from patients residing in a low or high gastric cancer risk region of Colombia. HEK-Blue-hTLR9 cells (TLR9$^+$) and HEK-Blue-Null1 (Parental) cells (Invivogen, San Diego, CA USA) were seeded in 96 well plates (Co-Star, St. Louis, MO USA) at 80,000 cells per well in DMEM (Corning, Manassas, VA USA) without antibiotics and were challenged with either viable *H. pylori* (MOI 100) or media alone at 37°C with 5% CO_2 for 24 hours. After 24 hours, 20μl of supernatants were added to 180μl of HEK-Blue Detection media (Invivogen). Plates were analyzed by spectrophotometer (Bitoek, Winooski, VT USA) at 650nm. All experiments were performed in duplicate and repeated at least 3 times. Data are expressed as fold over uninfected control. N=9 isolates per group, mean±SEM are shown. A one-way analysis of variance with Bonferroni correction was used to determine statistical significance between groups. **(b,c)** Gastric antral biopsy samples from patients residing in a high or low gastric cancer risk region in the Colombia, who were enrolled in an ongoing prospective study designed to study mechanisms of *H. pylori* carcinogenesis (57), were used for immunohistochemistry (histologic scores mean±SEM; low-risk, 2.05±0.11; high-risk, 3.89±0.26, p≤0.0001 Student's *t*-test; histology scored as previously described (57)). Immunohistochemistry was performed on paraffin-embedded biopsy samples from de-identified, *H. pylori*-infected, patients with non-atrophic gastritis, intestinal metaplasia (IM), or gastric dysplasia. Tissue

samples were deparaffinized and stained with a polyclonal anti-TLR9 antibody (1:1000, Imgenex San Diego, CA USA). A single pathologist (MBP) scored TLR9 IHC staining by assessing the percentage of $TLR9^{+}$ epithelial cells. Magnification 40x; Scale bar, 50μm. Epithelial TLR9 staining of biopsies obtained from patients in the low (N=11) and high (N=12) gastric cancer risk regions of Colombia is quantified in **(c).** Student's *t*-test was used to determine statistical significance between groups. **(a,c)** *p<0.05, **p<0.01,***p<0.001, ****p<0.0001.

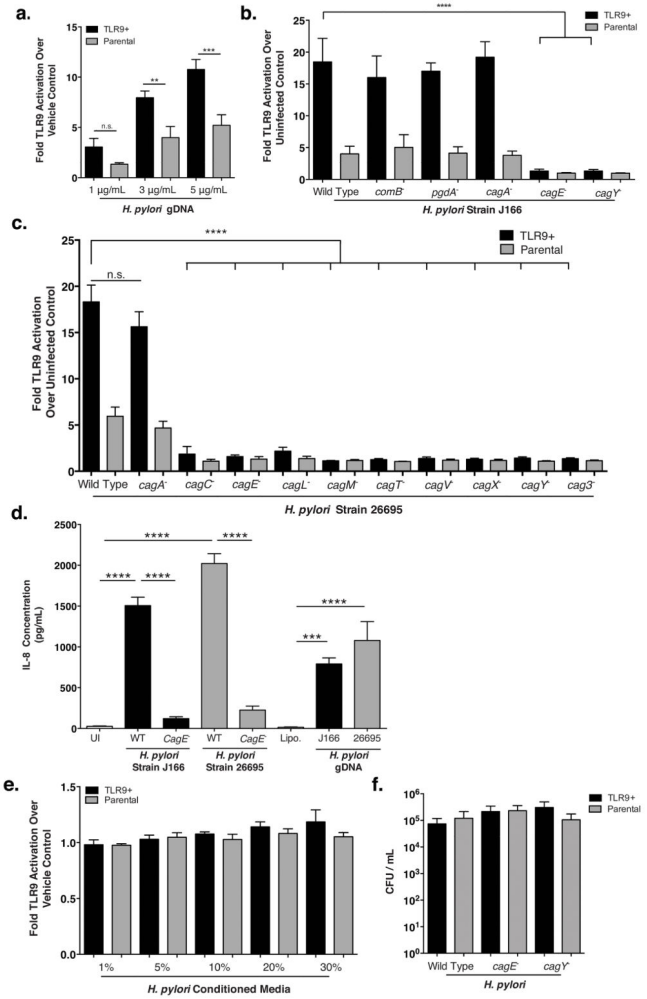

Figure 2. *H. pylori* activation of TLR9 requires a functional *cag* T4SS

(a–c) The *H. pylori cag*$^{+}$ strains J166 and 26695 (ATCC 700392) were maintained on trypticase soy agar plates supplemented with 5% sheep blood (BD Biosciences, Sparks MD USA) as described (48, 58). Isogenic mutants were constructed as previously described (48, 55, 58–62). Flanking sequences for *comB* were amplified from *H. pylori* strain J166 DNA using primers *comB8* Forward (5′-ACTAGAGCTCAAGCCTTTCAATAGCGAGCA- 3′), *comB8* Reverse (5′-AGTACCGCGGAGCGATTTTCAAGCGGTTC -3’) and *comB10* Forward (5’-CTGAGAATTCTTGCAATTGATGAGGCAAAG-3′) *comB10* Reverse (5′-ACTAGGTACCGCGATGACTTCATTCTCTCTGG -3′). *comB* flanking sequences were cloned into a pBSC103 plasmid using a previously inserted kanamycin resistance cassette generated by restriction enzymes *SacI* and *SacII* (*comB8*) and *EcoRI* and *KpnI* (*comB10*). The resultant plasmid was used to transform *H. pylori* strain J166 and transformants were selected on Brucella agar plates supplemented with kanamycin (5 μg/mL). Correct orientation of the kanamyacin cassette with *H. pylori comB* was confirmed by PCR analyses. **(a)** *H. pylori* strain J166 DNA was purified by growing microbial cultures in Brucella broth supplemented with 10% neonatal calf serum (NCS) (Atlanta Biologicals, Atlanta, GA USA) overnight. Cultures were centrifuged (4000 RPM, 5 min), resuspended in

600 μL of TE buffer with 0.5% SDS and 100 μg/mL proteinase K (Qiagen Germantown MD USA), and incubated at 37°C for 1 hour. DNA was extracted using CTAB and purified by phenol-chloroform extraction. TLR9-reporter or parental cells were cultured as described in Figure 1 and challenged with purified *H. pylori* strain J166 DNA (1–5 μg/mL) supplemented with Lipofectamine 2000 (Life Technologies, Carlsbad CA, USA) at 37°C with 5% CO_2 for 24 hours. Data are represented as fold over vehicle control. **(b)** TLR9 activation induced by *H. pylori* strain J166 or its isogenic mutants (MOI 100, T=24hrs), relative to uninfected control. **(c)** TLR9 activation induced by *H. pylori* strain 26695 or its isogenic mutants (MOI 100, T=24hrs), relative to uninfected control. **(d)** AGS gastric epithelial cells (ATCC) were grown in RPMI (Cell Gro, Manassas, VA, USA) supplemented with 5% fetal bovine serum (Atlanta Biologicals). Cells were seeded at 500 000 cells per well in a 12-well culture dish (Corning) and infected with either *H. pylori* strains J166 or 26695 (MOI 100), their respective *cagE*$^{-}$ mutants (MOI 100), or their respective purified gDNA (5 μg/mL) for 6 hours. Levels of IL-8 were quantified using Human CXCL8 ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions and tested in duplicate. **(e)** Liquid cultures of *H. pylori* were grown with shaking overnight in 5 mL of Brucella broth (BD Biosciences) supplemented with 10% neonatal calf serum (NCS) (Atlanta Biologicals) at 37°C and 5% CO_2. Supernatants of overnight cultures were collected, filtered (0.45 μm filter) and used for TLR9 activation assays at concentrations ranging from 1–30%. Results are shown relative to vehicle (Brucella broth) control. **(f)** HEK293 reporter cells were co-cultured with *H. pylori* wild type or isogenic mutant strains of J166 at an MOI of 100 for 4 hours. Cells were washed 3 times with PBS containing gentamicin (250 μg/mL; Corning). Cells were then incubated at 37°C for an additional hour in RPMI containing gentamicin (250 μg/mL), washed 3 times with PBS, lysed in 200 μL of sterile dH2O and serial dilutions were plated on TSA plates with 5% sheep blood (BD Biosciences). Plates were incubated for 5 days at 37°C, 5% CO_2 and colonies were enumerated. Experiments were repeated at least 3 times. Viable colony-forming units with mean±SEM are shown. Student's *t*-test **(a)** or one-way analysis of variance with Bonferroni correction **(b–f)** was used to determine statistical significance between groups. *p<0.05,**p<0.01,***p<0.001, ****p<0.0001.

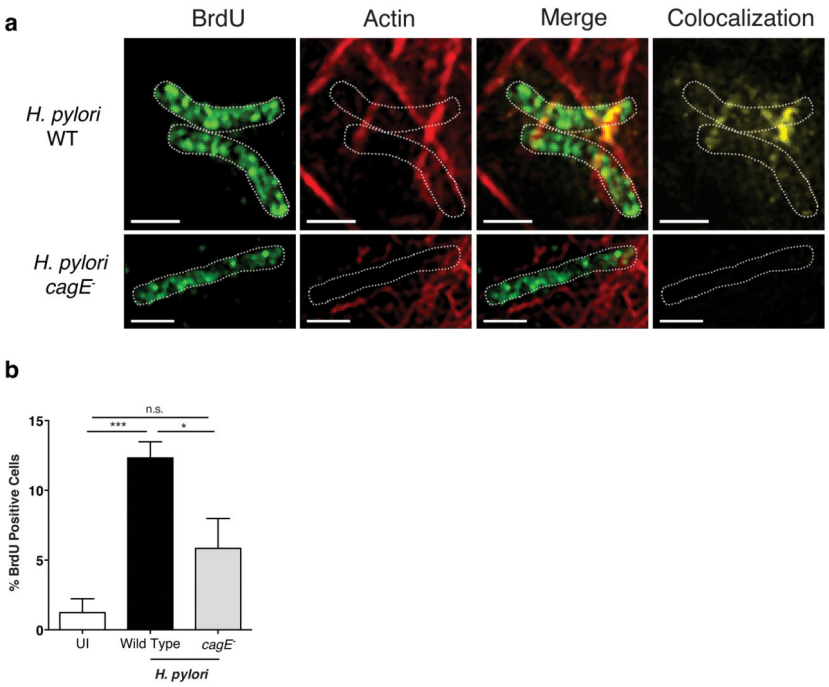

Figure 3. *H. pylori* translocates DNA into host cells via the *cag* T4SS

(a–b) *H. pylori* were grown overnight in Brucella broth containing 10% NCS. *H. pylori* were then diluted 1:10 in Brucella broth containing 5 μM BrdU (Sigma-Aldrich, St. Louis MO USA) and 10% NCS. Cultures were grown for an additional 4 hours prior to co-culture with eukaryotic cells. **(a)** *H. pylori*-mediated DNA translocation was assessed by structured illumination microscopy using AGS gastric epithelial cells co-cultured with BrdU-labeled *H. pylori* wild-type strain J166 or the J166 isogenic *cagE*$^{-}$ mutant. Scale bar, 1 μm. AGS cells were seeded on size 1.5 cover slips at 100 000 cells per well. BrdU-labeled *H.* were co-cultured at an MOI of 100 for 4 hours. Infected cells were washed 3 times in PBS, fixed with CytoFix/CytoPerm (BD Biosciences) solution for 20 minutes at 4°C and washed an additional 3 times in 1× permwash (BD Biosciences). Cells were then blocked overnight at 4°C using endogenous biotin blocking kit reagent A (Life Technologies), washed 3 times in 1× permwash and blocked again with endogenous biotin blocking kit reagent B (Life Technologies) for 30 minutes at room temperature. Cells were incubated with mouse anti-BrdU conjugated biotin (Life Technologies 1:500) overnight at 4°C diluted in 1× permwash. Cells were subsequently stained with strepdavidin-conjugated AlexaFluor488 (1:5000 for BrdU) as well as phalloidin (1:100 actin, Life Technologies) and Hoechst (1:5000 nuclei, Life Technologies) for 1 hour at room temperature. Cells were washed 3 times in 1× permwash and slides were mounted with prolong gold (Invitrogen). Slides were viewed with Delta Vision OMX confocal microscope (GE Health Care Life Sciences, Marlborough MA USA) at 63X magnification, using 1.514 immersion oil. Post-data acquisition processing was performed using SoftWorx for OMX. Images are shown as maximum intensity projections through the entire imaged area. BrdU, green; actin, red; merge, yellow. *H. pylori* are outlined by dotted white lines. Scale bar, 1μm. **(b)** BrdU-labeled *H. pylori* wild-type strain J166 or the J166 *cagE*$^{-}$ mutant were co-cultured with AGS cells and then subjected to flow cytometry to assess levels of host intracellular BrdU. AGS cells were co-cultured with

BrdU-labeled *H. pylori* for 4 hours. Cells were washed and incubated for an additional hour in RPMI 1640 containing gentamicin (500 μg/mL). Adherent cells were then dissociated with 0.25% trypsin EDTA (Life Technologies) supplemented with gentamicin (500 μg/mL), fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences), blocked overnight at 4°C using endogenous biotin blocking kit reagent A (Life Technologies), washed 3 times in 1× permwash (BD Biosciences) and blocked again with endogenous biotin blocking kit reagent B for 30 minutes at room temperature (Life Technologies). Cells were washed and stained with biotin conjugated anti-BrdU mouse monoclonal antibody (1:500 Life Technologies) followed by strepdavidin conjugated AlexFluor488 secondary antibody (1:5000 Life Technologies). Cells were acquired using a LSR II Flow Cytometer (BD Biosciences) and BrdU-positive cells were analyzed using FlowJo (Tree Star Inc. Ashland OR USA). Each strain was tested at least 3 times. Mean±SEM are shown. *$p<0.05$, ***$p<0.001$.

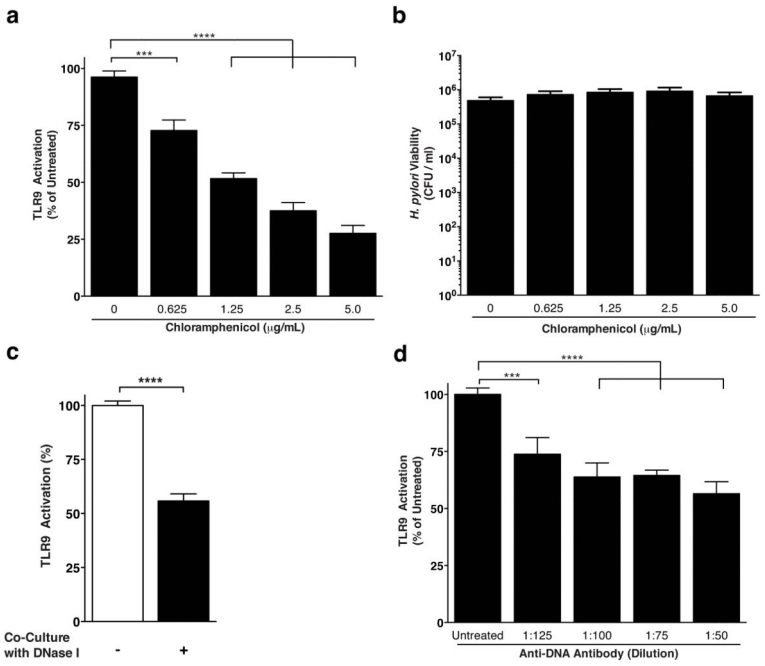

Figure 4. Mechanisms of *H. pylori cag*-mediated DNA translocation

(a) *H. pylori* strain J166 was inoculated into HEK-Blue-hTLR9 and HEK-Blue Null1 co-cultures as described in Figure 2 legend. For each biological replicate experiment, sub-lethal levels of chloramphenicol (Sigma-Aldrich) were added to triplicate wells at concentrations ranging from 0.625 μg/mL to 5 μg/mL. After 24 hours, TLR9 activation was assessed as described for TLR9 activation assays. For each concentration, OD_{650} values obtained for HEK-Blue-hTLR9 cells were normalized by subtracting the corresponding OD_{650} values obtained for HEK-Blue-Null1 cells, and TLR9 activation was calculated as a percent of untreated, antibiotic-free co-cultures. **(b)** After 24 hours of co-culture in chloramphenicol, *H. pylori*-HEK co-cultures wells were dislodged by mechanical disruption, centrifuged at 5000 *x g*, and were re-suspended in 1 mL sterile PBS. To calculate *H. pylori* viability in CFUs, 10-fold serial dilutions of resuspended co-cultures were plated, and colonies were enumerated after 72 hours of incubation. **(c)** *H. pylori* wild-type strain J166 was co-cultured with HEK-Blue-hTLR9 reporter cells at an MOI of 100. For the course of the co-culture, 20 units of Turbo DNase I (Life Technologies) were added to half of the wells, and DNase I buffer was added to the remaining wells. TLR9 activation was assessed at 24 hours. For each condition, OD_{650} values obtained for HEK-Blue-hTLR9 cells were normalized by subtracting the corresponding OD_{650} values obtained for HEK-Blue-Null1 cells, and TLR9 activation was calculated as a percent of untreated, DNase I-free co-cultures. **(d)** *H. pylori* strain J166 was inoculated into HEK-Blue-hTLR9 and HEK-Blue-Null1 co-cultures at an MOI of 100. For each biological replicate experiment, monoclonal anti-DNA antibody (Roche, Mannheim Germany) was added at varying dilutions, ranging from 1:50 – 1:200 final concentration. After 24 hours, TLR9 activation was assessed, and values were normalized as described for DNase I experiments. TLR9 activation was expressed as a percentage of untreated (no antibody) wells. Experiments were repeated at least 3 times. For all experiments, mean±SEM are shown. A one-way analysis of variance with Bonferroni

correction was used to determine statistical significance between groups. *p<0.05,**p<0.01,***p<0.001, ****p<0.0001.