Supporting Online Material

Materials and Methods:

Cell and bacterial culture

Routine culture of MDCK, AGS cells, and *H. pylori* was as described previously (*1*). *H. pylori* were also grown in continuous co-culture with MDCK confluent monolayers using DMEM 10% FBS (Gibco, BRL), 10% Brucella broth, 10μg/ml vancomycin (Sigma). *H. pylori* grown on MDCK monolayers were maintained in log phase by replacing the media daily, maintaining CFU's between 10^6 and 10^8 bacteria/ml. For cell infections, *H. pylori* were grown on MDCK monolayers to approximately 10^8 bacteria/ml. Spiral, rapidly-swimming bacteria were used for infection. Isogenic strains of *H. pylori* utilized in this manuscript are listed in Table S1. For confocal immunofluorescence cells were grown on glass coverslips, infected with *H. pylori* for different periods of time and fixed with 2% paraformaldehyde in 100mM phosphate buffer pH 7.4. Cells were permeabilized in PBS 1% saponin, 3% bovine serum albumin (BSA). After staining with appropriate antibodies, samples were imaged with a BioRad confocal microscope and the z-stacks were projected onto z-projections or 3D reconstructions using NIH Image-J or Volocity 2.0 software. Figures were assembled with Photoshop 7.0. Antibodies used in the manuscript are described in Table S2.

Tight junction barrier function

To assess for solute leakage across the epithelial monolayer after *H. pylori* infection, MDCK cells were grown as a confluent polarized monolayer on Transwell- Polyester Membrane filters (Costar) for 3 days and then infected in the apical chamber with *H. pylori*. The infecting *H. pylori* were co-cultured with MDCK for 10 days before addition of biotinylated albumin (Sigma) at 20μg/ml to the media in the basal-lateral chamber. The apical chamber media was sampled 1 hr and 24 hrs later and equal amounts of media from each sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The presence of biotinylated albumin leaking from the basolateral chamber was detected by incubating the blots with streptavidin conjugated to Alexa Fluor-660 and fluorescence detection using the Licor-Odyssey scanner. To detect dysfunction of individual junctions, confluent MDCK monolayers grown on permanox dishes (Lab-Tek) were infected for 3 days with *H. pylori* and then fixed in 2% glutaraldehyde 100 mM sodium cacodylate buffer, pH 7.4, containing 0.2% ruthenium red. After post fixation in 1% osmium tetroxide with 0.2% ruthenium red the samples were processed for TEM. Sections were imaged in a Phillips CM-12 to look for evidence of ruthenium red leakage from the apical surface into the intercellular spaces.

Synchronization of Junction formation by calcium-switch

To determine the effect of CagA on the formation of mature tight junctions, MDCK monolayers were synchronized for junction formation by calcium-switch experiments (*2*). Briefly cells are plated in DMEM/ 10% FBS medium containing 5 μm calcium onto collagen coated coverslips. After attachment, cells are transferred into DMEM/ 10% FBS

medium containing 1.8 mM calcium to initiate E-cadherin-dependent junction formation and bacteria are added simultaneously.

Membrane fractionation by Iodixanol Gradients

Separation of different membrane compartments was performed a described before (*3*). Briefly, cells were cross-linked with DSP (Dithiobis[succinimidylpropionate], Pierce) prior to mechanical breakage of cells. Postnuclear supernatant of mechanical broken cells was mixed with equal amounts of Opti-Prep™ (60% (wt/vol) iodixanol (Greiner Bio-One)) and overlayed with equal volumes of 20% and 10% iodixanol in homogenization buffer in centrifuge tubes. After centrifugation at 350,000xg for 3 hrs at 4°C in Beckman Coulter VTI 65.1 rotor, fractions (0.5 ml) were collected and proteins were separated by SDS-PAGE and immunoblotted. Density of each fraction was determined by refractometry.

Supplementary Tables

Strain Name	Name in manuscript	Description
G27	Wild Type	Clinical isolate from a patient with peptic ulcer disease (*4*).
G27-MA-3/25-4	MDCK-adapted Wild Type or G27-MA	Natural variant of G27 selected for increased adhesion and ability to deliver CagA to MDCK cells (see Fig. S1).
G27-MA-3/25-4-Δ*cagA*-1	MDCK-adapted ΔCagA	Generated by deletion insertion of a chloramphenicol resistance cassette into the cagA gene as described in (*1*). Disruption of the gene was confirmed by PCR and immunoblotting with anti-CagA antibodies.
G27-ΔCagA	ΔCagA	Generated by deletion insertion of kanamycin and *sacB* genes in the *cagA* gene as described in (*5*).
G27-CagA*	CagA*	Complemented wild type strain made from G27-Δ*cagA* by homologous recombination of the *cagA* gene as described (*5*).
G27-$CagA_{EPISA}$	EPISA, $CagA_{EPISA}$	Expresses mutant CagA that cannot be tyrosine phosphorylated as described (*5*).
G27-Δ*cag7,* Δ*virB10*	G27-Δ*virB10* (Δ*cag7*)	Deletion of one of the TFSS genes essential for CagA translocation. Made by deletion insertion as described (*5*).
*G27-*Δ*cag23*, Δ*virB4*	*G27-*Δ*virB4* (*cag23*, *cagE*)	Deletion of one of the TFSS genes essential for CagA translocation. Made by deletion insertion as described (*6*).
G27-GFP pTM115	G27-GFP	G27 tranfected with plasmid pTM115 that contains the enhanced green fluorescent protein (eGFP) gene under control of the urease promoter.

Table S1. *H. pylori* strains

Antibody or Reagent	Figures	Clone or Serum	Description or Vendor
Mouse anti-*H. pylori*	1b- red, 1c-red, 2a-red, 2b-red, S1d-red, S2a-red	Anti *Helicobacter pylori*, clone 51-13	Murine IgG1. From Monosan, Uden, TheNetherlands
Chicken anti-*H. pylori*	1a-red, 4f-blue, S2b-green	Chicken polyclonal antibody solution anti-*H. pylori* #67	Made against paraformaldehyde fixed *H. pylori* strain G27-MA-3/25-4-Δ*cagA*
Rabbit pAb anti-ZO-1	1a-c-green, 2a-green, 2b-green, 4a-d,f-green, 4b,c-inset red, 4f-green, S1d-green, S2a-green, S2b-blue	Rabbit anti-ZO-1 Cat #61-7300	Zymed Laboratories, South San Francisco, CA
Mouse mAb anti- ZO-1	2c-blue, 4e,g-blot	Mouse anti-ZO-1 Cat # 33-9100	Zymed Laboratories, South San Francisco, CA
Mouse mAb anti- human JAM-1	4f-red	Anti-hJAM murine mAb IgG1	Obtained from Dr. Charles A. Parkos
Rabbit anti-JAM pAb	4e-blot	pAs #1097 affinity purified	Made against the extracellular domain of JAM expressed in *E. coli*. Immune serum recognizes the 40 kD band of human JAM detected by monoclonal anti-JAM antibodies. Serum was affinity purified using recombinant JAM protein.
Mouse anti-E-cadherin	1a-blue	3G8 mouse monoclonal antibody, specific for the extracellular domain of canine E-cadherin	Referenced in (*7*)
Rabbit anti-CagA pAs	2c-red, S2b-red, S2c- green blot	Rabbit polyclonal antiserum anti-CagA N2	Made against full-length recombinant CagA expressed in *E. coli* (DH10B at 30°C) and purified by chromatography and SDS-PAGE.
Mouse anti-CagA mAb for immunoblots	4e,g-blot, S1b-blot, S2c-red blot	HPM-5001-5. Anti-Helicobacter Cag antigen	Austral Biologicals. San Ramon, CA
Mouse anti-phosphotyrosine mAb	4g-blot, S1b-blot	P-Tyr-1000 #9411	Cell Signaling Technology. Beverly, MA
Mouse anti-SHP2 mAb	4e-blot	S-PTP2 (B-1): sc-7384 affinity purified IgG1 to carboxy teruminus of human SHP2	Santa Cruz Biotechnology, Inc., CA
Rabbit anti-occludin pAb	4e-blot	Rabbit anti-ZO-1 Cat #71-1500	Zymed Laboratories, South San Francisco, CA
Alexa Fluor 660 phalloidin	4d-orange, 2Sa-blue	A-22285 Alexa Fluor® 660 phalloidin	Molecular Probes. Eugene, OR
Streptavidin Alexa Fluor® 660	3a- blot	S-21377 streptavidin, Alexa Fluor® 660 conjugate	Molecular Probes. Eugene, OR
Goat anti-mouse Alexa Fluor® 594 (red)	1b,c-red, 2a,b-red, 4f-red, S2a-red	A-11005 Alexa Fluor® 594 goat anti-mouse IgG (H+L) conjugate	Molecular Probes. Eugene, OR
Goat anti-rabbit Alexa Fluor® 488 (green)	1a-c-green, 2a,b-green, 4a-d,f-green, S2a-green	A-11008 Alexa Fluor® 488 goat anti-rabbit IgG (H+L) conjugate	Molecular Probes. Eugene, OR
Goat anti-mouse Alexa Fluor® 660 (far red)	1a-blue, 2c-blue, S2b-blue, S2c-red	A-21054 Alexa Fluor® 660 goat anti-mouse IgG (H+L)	Molecular Probes. Eugene, OR
Goat anti-rabbit Alexa Fluor® 594 (red)	2c-red, 4b,c inset-red, S2b-red	A-11012 Alexa Fluor® 594 goat anti-rabbit IgG (H+L) conjugate	Molecular Probes. Eugene, OR
Goat anti-chicken Alexa Fluor® 488 (green)	S2b-green	A-11039 Alexa Fluor 488 goat anti-chicken IgG (H+L) conjugate	Molecular Probes. Eugene, OR
Goat anti-rabbit IRDye800	S2c-green	611-132-122 Goat anti-rabbit IRDye800 IgG (H+L) conjugate	Rockland Immunochemicals, Gilbertsville, PA

Table S2. Antibody Reagents

Supplementary Figures

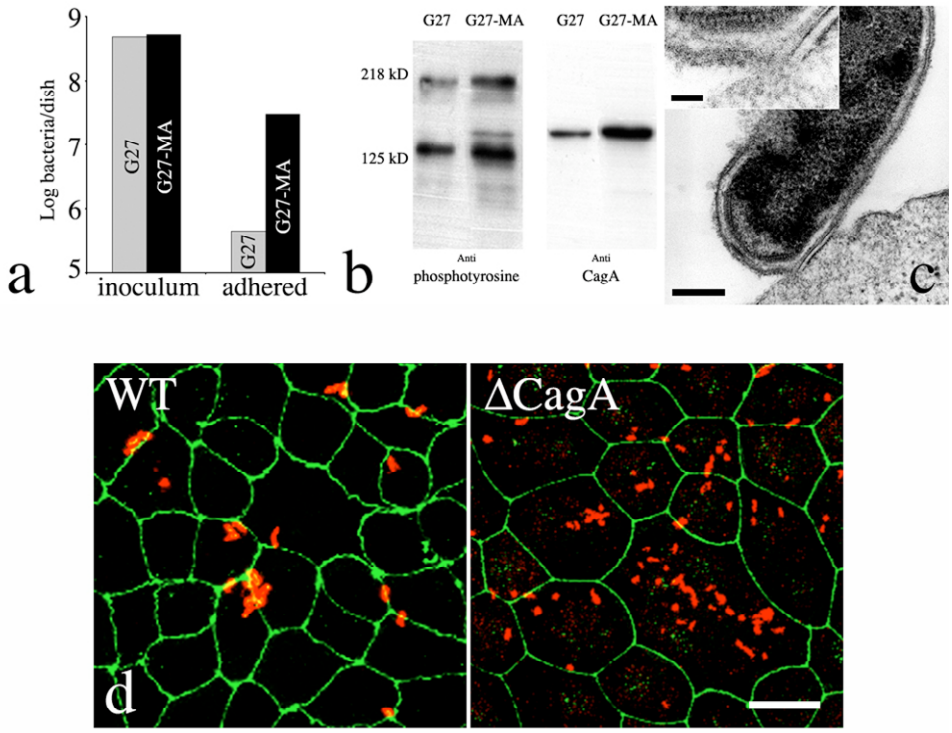

Figure S1. **Isolation of MDCK-adapted G27 *H. pylori*.** A co-culture system of *H. pylori* and MDCK cells was developed in which *H. pylori* can be maintained on the surface of a confluent MDCK monolayer for an indefinite period of time. Initially, *H. pylori* G27 adhered poorly to the surface of MDCK cells and no evidence of CagA delivery was detected. We cultured G27 *H. pylori* continuously for 4 months over an MDCK monolayer and enriched for *H. pylori* with increased adhesion to the monolayer. Adherent bacteria were isolated and passaged as single clones on blood agar plates. Each clone was tested for its ability to deliver CagA to MDCK cells in phosphotyrosine immunoblot assays. One clone (3/25-4, G27-MA) with increased adherence to MDCK and which delivers CagA to these cells is used in this study. **a**) Representative adherence assay- Equal inocula (by CFU) of *H. pylori* G27 or MDCK-adapted G27 (G27-MA) were allowed to attach for 3 hours to the surface of MDCK monolayers grown on glass coverslips. The monolayers were washed free of unattached bacteria, fixed and stained with anti-*H. pylori* antibodies. The number of bacteria adhered to the cells were counted in 10 random fields by immunofluorescence microscopy. The average number of bacteria/field (G27-5.4±1.1 vs. G27-MA 365.4±26.7) was normalized to the total dish area. **b**) Detection of tyrosine phosphorylated CagA by immunoblot. MDCK monolayers were incubated with parental G27 or MDCK-adapted *H. pylori* (G27-MA) for 8 hours. Lysates were separated by SDS-PAGE and immunoblotted with anti-CagA and anti-phosphotyrosine antibodies. **c**) Transmission electron micrograph (TEM) of *H. pylori* adhered to the surface of a MDCK cell showing close apposition of bacterial and host cell membranes. Bar 0.25 μm. Inset: site of contact. Bar 50 nm. **d**) Confocal immunofluorescence z-projections of MDCK confluent monolayers infected with wild type MDCK-adapted *H. pylori* (WT) or ΔCagA *H. pylori* for 30 minutes, washed, and incubated for 8 hours, prior to fixation and staining with anti-ZO-1 (green) and anti-*H. pylori* (red) antibodies.

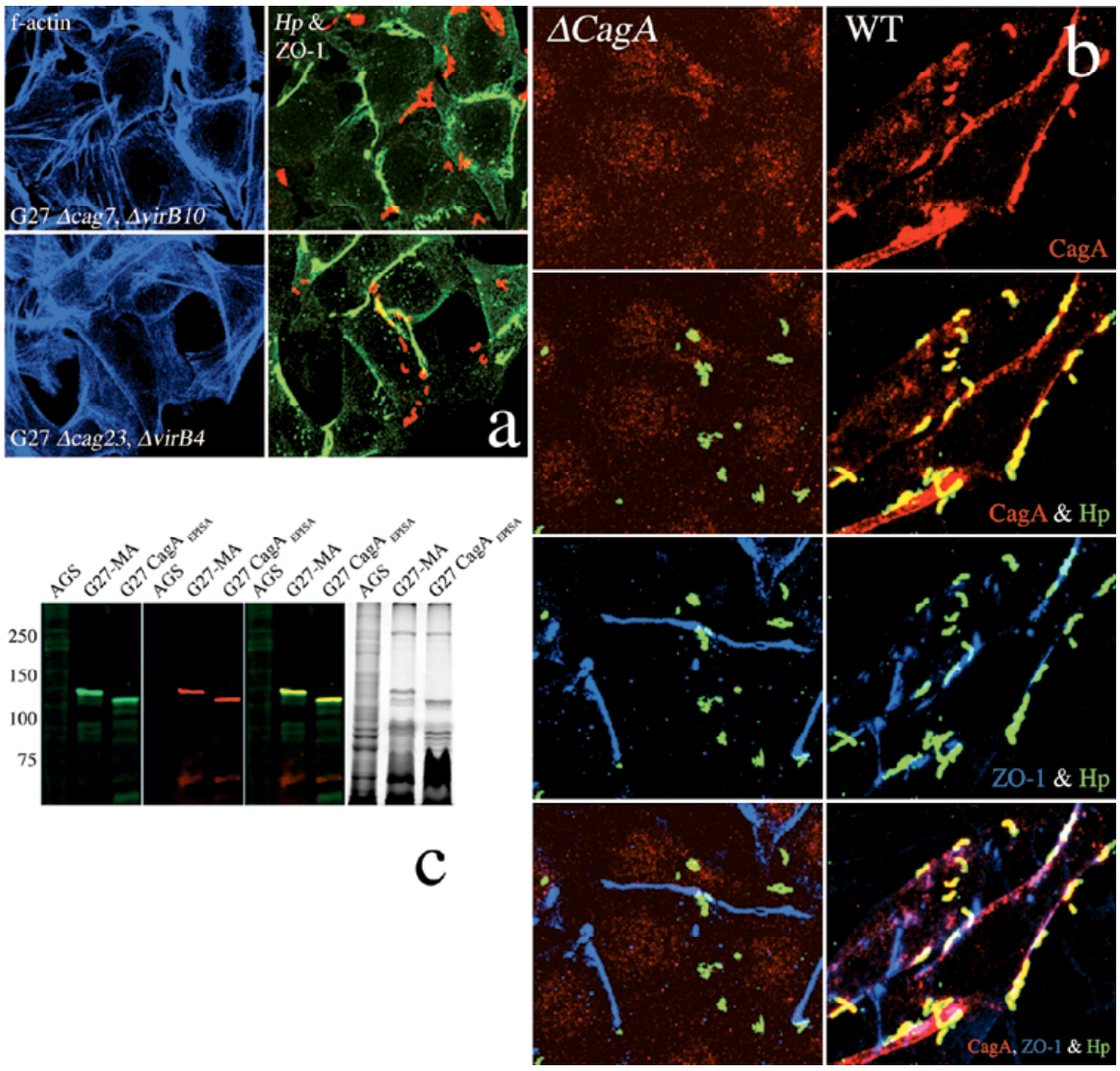

Figure S2. **a**) **TFSS is necessary for *H. pylori* association with ZO-1**. Confocal immunofluorescence of AGS cells infected for 5 hours with G27 *H. pylori* isogenic mutants of *virB10* homologue (Δ*cag7,* Δ*virB10*,) and *virB4* homologue (Δ*cag23,* Δ*virB4*). Anti-*H. pylori* antibodies (red), anti-ZO-1 (green) and f-actin stained with fluorescent phalloidin (blue). Note lack of co-localization between *H. pylori* and ZO-1, and lack of cellular elongation. **b, c**) **Specificity of polyclonal antiserum against CagA.** Triple staining confocal microscopy of CagA (red), ZO-1 (blue) and *H. pylori* (green) in infected AGS cells. Monolayers of AGS cells were infected for 3 hours with either wild type G27 *H. pylori* (WT, right column) or ΔCagA G27 isogenic strain (left column) and fixed in 2% paraformaldehyde phosphate buffer. Cells and bacteria were permeabilized with 1% saponin, 0.1% triton and 3% FBS in PBS prior to immunostaining. Anti-*H. pylori* polyclonal antibodies were prepared in chickens (green). Anti-CagA antibodies were prepared in rabbits against purified whole length recombinant CagA and pre-adsorbed with fixed Δ*cagA* G27 *H. pylori*. **c**) Immunoblotting of uninfected AGS cells, G27 *H. pylori* and G27 $CagA_{EPISA}$ lysates show specificity of rabbit polyclonal anti-CagA antibodies (green), compared to commercially available anti-CagA monoclonal antibodies (red, Austral Biologicals). An overlay of two antibody channels is shown on the third panel. Coomassie blue stained gel shows amounts of protein loaded. Western blots were analyzed using the Odyssey™ Infrared Imaging System (Li-Cor).

References

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