Cag3 is a novel essential component of the *Helicobacter pylori* Cag Type IV secretion system outer membrane subcomplex

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Supplemental Material

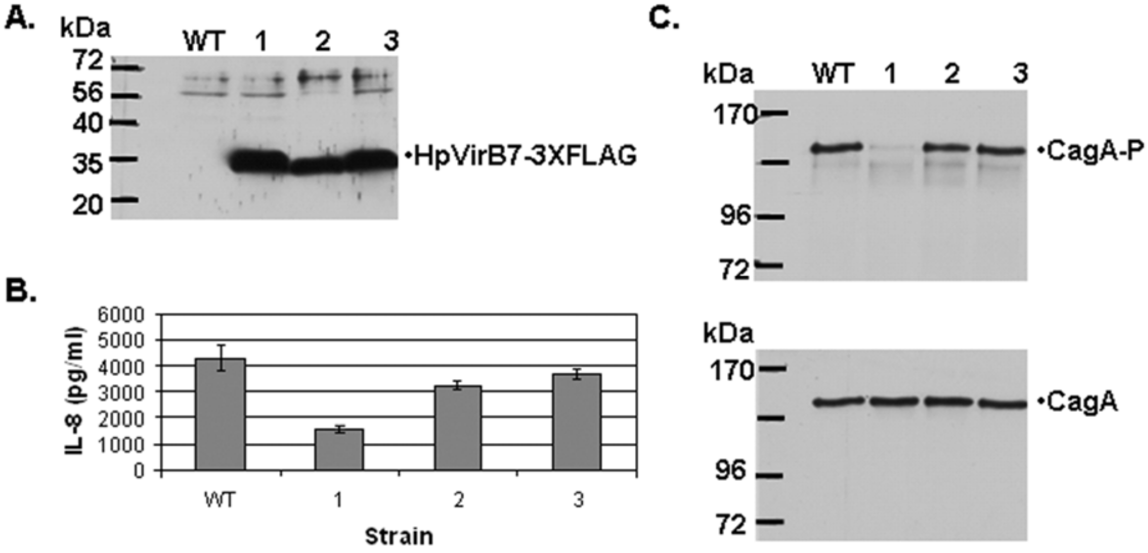

Figure S1. *H. pylori* strains expressing HpVirB7-3XFLAG retain Cag T4S activity. A. Immunoblot analysis of total protein extracts from WT (NSH57 not expressing HpVirB7-3XFLAG) and strains 1, 2, and 3 (chloramphenicol resistant clones predicted to express the HpVirB7-3XFLAG) separated by 10% SDS-PAGE and probed with anti-M2 antibody to detect HpVirB7-3XFLAG. Molecular weight markers sizes in kDa are indicated. As shown strains 1, 2 and 3 expressed the predicted 35 kDa HpVirB7-3XFLAG. **B.** Induction of IL-8 secretion. Media harvested from a co-culture experiment with AGS cells for 24 hours at a multiplicity of infection of 10:1 (in triplicate) was used for determination of secreted IL-8 by ELISA. Means from a representative experiment are shown. Error bars indicate one standard deviation. **C.** Total protein extracts from same co-culture experiment used in B. were separated by 6% SDS-PAGE and probed with α-mouse 4G10 (upper panel) to detect CagA phosphorylation and subsequently probed with α-rabbit CagA antibody to detect total CagA (lower panel). Strains 2 and 3 were able to sustain wild-type Cag T4SS as seen by their ability to induce IL-8 secretion and host catalyzed CagA phosphorylation. Strain 3 (NSH57, *HpVirB7::3xFLAG::cat*) was used for all subsequent experiments in this study.

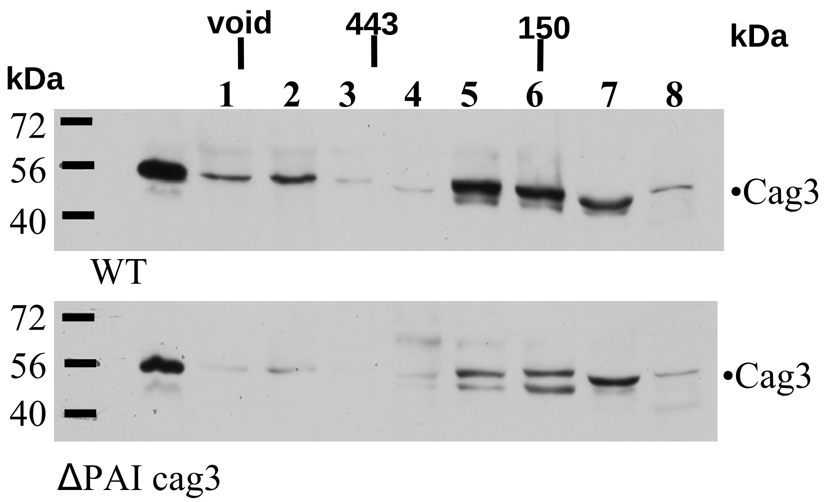

Figure S2. Cag3 migrates primarily in a single peak in a strain lacking the *cag*PAI. Whole cell extracts of WT (upper panel) and *ΔPAI cag3* (lower panel) strains were fractionated on a SuperDex 200 gel filtration column and the presence of Cag3 in each fraction was determined by immunoblotting with α-rabbit-Cag3. Molecular weight markers sizes in kDa are indicated. Fraction 1 is the void volume at which Dextran Blue eluted, the 443 kDa marker eluted in fraction 3 and the 150 kDa marker eluted in fraction 6. WT: *HpVirB7-3XFLAG::cat*; ΔPAI cag3: *ΔcagPAI::aphA3 rdxA::cag3*.

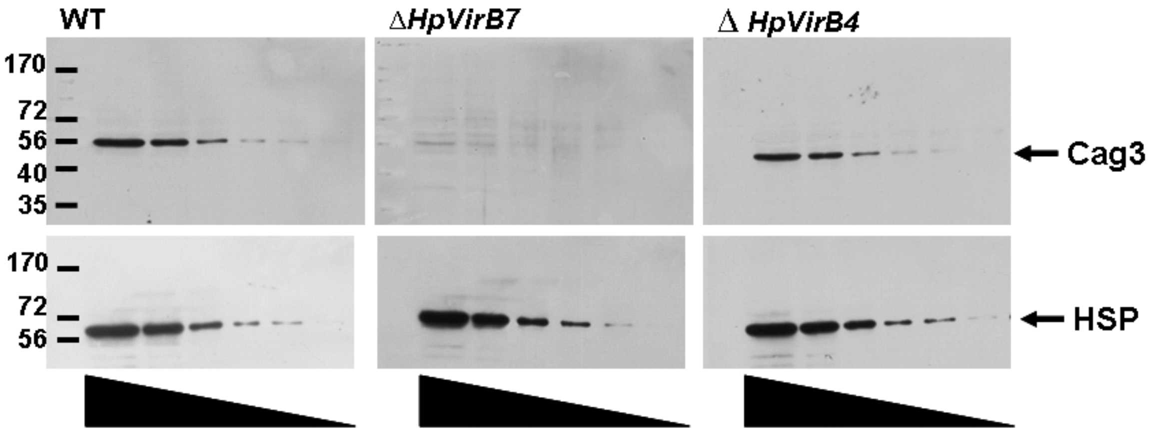

Figure S3. Cag3 steady state levels are reduced in a Δ*HpVirB7* strain. Equal amounts of *H. pylori* cells from the indicated strains were washed with PBS and resuspended in same volume of 2.5% SDS buffer. Serial three fold dilutions of whole cells extracts were separated by 10% SDS-PAGE and Cag3 steady state levels were assessed by immunoblotting with α-rabbit-Cag3 antibody. Immunoblots were stripped and reblotted with α-mouse-HSP antibody as a loading control. Molecular weight markers sizes in kDa are indicated. WT: wild-type; ΔHpVirB7: *HpvirB7::cat;* ΔHpVirB4: *HpvirB4::cat* .

Table S1. Predicted T4S system components encoded in the *H. pylori cag*PAI

H. pylori 26695 annotation	Predicted signal peptide	*A. tumefaciens* homologue	Putative function and/or subcellular location	Length[a] in *H. pylori*	Length[a] in *A. tumefaciens*
HP0523	yes	VirB1	Transglycosylase	169	239
HP0524	no	VirD4	Inner membrane ATPase	748	668
HP0525	no	VirB11	Inner membrane ATPase	330	343
HP0527	no	VirB10	Inner and outer membrane	1927	377
HP0528	yes	VirB9	Outer membrane Secretin-like	522	293
HP0529	no	VirB6	Inner membrane	535	295
HP0530	no	VirB8	Inner membrane	252	237
HP0532	yes	VirB7	Outer membrane lipoprotein	280	52
HP0539	yes	VirB5	Pilus-associated adhesin	237	220
HP0544	yes	VirB4	Inner membrane ATPase	983	789
HP0546	yes	VirB2	Main pilus subunit	115	121

[a] total number of amino acid residues

Table S2. Oligos used in this study.

Name	Sequence
5’N1cag3 oligo 1	5’ CACAAGCGACCTATAAAATGATAC 3’
3’C1cag3stopcat oligo 2	5’ACCGCTGTAT**A**GCTCATAGGT**gatatagattgaaaagtggat** 3’
5’N2cag3 oligo 3	5’**cccagtttgtcgcactgataa**TTACTAACCTCTAACACTCTT 3’
3’C2 cag3 oligo 4	5’CAACAACTATTTATCCATAGAAAAC3’
3’C1cag3stopkan oligo 2	5’**acagaataactctatgaagcg**ACCGCTGTAT**A**GCTCATAGGT 3’
5’N2cag3Kan oligo 3	5’**atctaggtactaaaacaattc**TTACTAACCTCTAACACTCTT 3’
HpVirD4 oligo 1	5’GCTCTAGAGTCTTATTGAGGGCTTTAAAG 3’
HpVirD4 oligo 2	5’GTGGGTTCAAGTGAACTGTGA*gtcgac*GCGT 3’
HpVirD4 oligo 3	5’**atccacttttcaatctatatc**TATAGATGGGCTGAAAGAAAG 3’
HpVirD4 oligo 4	5’**cccagtttgtcgcactgataa**GGTAGGAATGGCGCTAAGACT 3’
HpVirB11 oligo 1	5’g*ctctag*AAAAGAGCTAAATTGATAACCC 3’
HpVirB11 oligo 2	5’acgc*gtcgac*AATAACGCTATTAACCCTATG 3’
HpVirB11 oligo 3	5’**atccacttttcaatctatatc**GCTCGGTGTTGTGCAAGTTTT 3’
HpVirB11 oligo 4	5’**cccagtttgtcgcactgataa**CATACGATTTTTATAATGTGC 3’
HpVirB10 oligo 1	5’ *gctcta*gaAAACTATGGTGAATTGGAGCG 3’
HpVirB10 oligo 2	5’ GGCTCTTCTAGAGGATATAGT*gtcgac*GCGT 3’
HpVirB10 oligo 3	5’ **atccacttttcaatctatatc**GTCTAGCAGACAAGTTATTCA 3’
HpVirB10 oligo 4	5’ **cccagtttgtcgcactgataa**GCAACTGATGAATATCCCCCC 3’
HpVirB9 oligo 1	5’ *gctcta*gaAATGGTTGGAAGAGTTGGTGG 3’
HpVirB9 oligo 2	5’ AAGCCCAACAAGATTCACCCC*gtcgac*GCGT 3’
HpVirB9 oligo 3	5’ **atccacttttcaatctatatc**GGTTGGAGTATTGTGCCTAAT 3’
HpVirB9 oligo 4	5’ **cccagtttgtcgcactgataa**TTTGTGGTTCAACCTGATGGG 3’
HpVirB6 oligo 1	5’ *gctctag*AGACGGTAAGCGAAATTTTGAG 3’
HpVirB6 oligo 2	5’ GTGGGTTCAAGTGAACTGTGA*gtcgac*GCGT 3’
HpVirB6 oligo 3	5’**atccacttttcaatctatatc**TATAGATGGGCTGAAAGAAAG 3’
HpVirB6 oligo 4	5’**cccagtttgtcgcactgataa**TTCAATTTGACCATAACGCCC 3’
HpVirB7 oligo 1	5’ G*ctctag*AAGGCGGTGCAGAATGAAACTA 3’
HpVirB7 oligo 2	5’ AGAGAGAAACGCCAACGGGCGGTCGACGCGT 3’
HpVirB7 oligo 3	5’ **atccacttttcaatctatatc**GATTACGCTCATAGGCGATGC 3’
HpVirB7 oligo 4	5’ **cccagtttgtcgcactgataa**TGGCAAGAGAAAAAGCTCAGC 3’
HpVirB4 oligo 1	5’ GCTCTAGATATACGATTACATGTGAAGCG 3’
HpVirB4 oligo 2	5’ GCGCGAACAAAAATTATTGAA*gtcgac*GCGT 3’
HpVirB4 oligo 3	5’ **atccacttttcaatctatatc**GCGATTGTTATTGTGCTTGTA 3’
HpVirB4 oligo 4	5’ **cccagtttgtcgcactgataa**TTTTTTTGAGAAACGATGGGG 3’
Ucag3Eco	5’GCGTTACTGAAAGATTCAAAG*gaattc*CGCA 3’
Cag3CSalI	5’*gtcgac*TACTTACCTTAAATGCAA 3’
Cag3Bam5-23	5’ACGCGGATCCAAAGAAATAAGTGAAGCCGAT 3’
Cag3EcoR1	5’GCGTTACTGAAAGATTCAAAGGAATTCCGCA 3’
CagMF	5’ TTTTGGATATGGAAAAATTTTG 3’
CagMR	5’ATTGATAGCGTTTAAGCCC 3’
Kan5’	5’ACAGAATAACTCTATGAAGCG 3’
Kan3’	5’ATCTAGGTACTAAAACAATTC 3’
B7F	5’CGC*gaatcc*ATCAATGGTCTTTTCACT 3’
B7FlagR	5’AGTTTGCTCAGTGGTAAG**GACTACAAAGACGATGA**

	CGACAAGTGA*gtcgac*GCTC 3’
B7F oligo1	5’CTTGCATGGCTATGATGTG 3’
B7-2XFLAG oligo 2	5’CAAAGACGATGACGA**CAAGGACTACAAAGACGATGACGACAAGGACTACAAAGACGATGACGACAAG**TGA**gatatagattgaaaagtggat 3’**
B7 oligo3	5’**cccagtttgtcgcactgataa**TTGGAAGAAAAAAGGGAGAG 3’
B7 oligo 4	5’GTTATATTGTTCAATGGTTTTTAG 3’

Italics indicates restriction site, Upper case bold indicates FLAG tag sequence, Underlined TGA indicates introduced stop codon, Lower case bold indicates sequence complementary to C. coli *cat* gene or *aphA* gene and bold A (oligo 3’C1cag3stopkan oligo 2) shows introduced nucleotide change to generate a premature stop codon.

Table S3. Cag3 interacting non-*cag* PAI proteins identified by mass spectrometry after anti-Cag3 affinity purification.

Gene Annotation[a]	Name/Function	Total peptides	Unique Peptides[b]	Prophet score
HP0319	arginyl-tRNA synthetase	2	2	0.99
HP0886	cysteinyl-tRNA synthetase	2	2	0.99
HP0658	Glu-tRNA(Gln) amido transferase subunit B	2	2	0.98
HP0601	flagellin A	5	5	0.97
HP0115	flagellin B	3	3	0.99
HP0703	transcriptional activator of flagella proteins	2	2	0.99
HP0751	putative polar flagellin	2	2	0.99
HP1156	outer membrane protein (omp25)	3	3	0.97
HP0896	outer membrane protein (omp19)	13	10	0.99
HP0198	nucleoside diphosphate kinase (ndk)	5	5	0.99
HP0162	hypothetical protein	2	2	0.99
HP0185	hypothetical protein	7	6	0.99
HP1042	hypothetical protein	2	2	0.99
HP0468	hypothetical protein	4	3	0.98
HP0385	hypothetical protein	2	2	0.97
HP0305	conserved hypothetical	2	2	0.95
HP0508	plasminogen binding protein	4	4	0.97
HP1550	secD	2	2	0.96
HP0298	dppA	2	2	0.97
HP0646	galU	2	2	0.99
HP1257	pyrE	2	2	0.99
HP0005	pyrF	2	2	0.99
HP0191	frdB	2	2	0.99
HP0723	L-asparaginaseII	2	2	0.99
HP0850	type I restriction enzyme M protein	2	2	0.97
HP0857	phosphoheptose isomerase	2	2	0.99
HP1088	transketolase	2	2	0.99
HP0691	succinyl-CoA-transferase subunit A	2	2	0.99
HP0692	succinyl-CoA-transferase subunit B	3	3	0.95

HP0557	accA, acetyl-coezyme A carboxylase	2	2	0.99
HP1137	ATP synthase F0. subunit b'	2	2	0.98
HP0516	hslU, heatshock protein ATP-binding subunit	4	4	0.99
HP0723	L-asparaginaseII	2	2	0.99
HP1088	transketolase	2	2	0.99
HP1362	replicative DNA helicase (dnaB)	2	2	0.99
HP1024	co-chaperone-curved DNAbinding protein A	2	2	0.95
HP0615	DNA ligase	4	2	0.99
HP0275	ATP-dependent nuclease (addB)	3	3	0.99
HP0391	CheA-MCP coupling protein	2	2	0.99
HP1186	alpha-carbonic anhydrase	4	3	0.99
HP0416	cyclopropane fatty acid synthase	2	2	0.99
HP1350	carboxyl-terminal protease	3	2	0.99
HP0416	Lipid biosynthesis	3	3	0.96
HP1375	lpxA	2	2	0.99
jhp1349	lpp20	5	5	0.97
HP0098	thrC	3	3	0.99
HP1040	ribosomal protein S15	2	2	0.99

[a] Gene annotation numbers from strain 26695 genome unless not, then J99 annotation numbers used (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). [b] Peptides identified in the eluate from wild-type extracts not detected in *cag3* mutant extract eluates.