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REVIEW

The *Helicobacter pylori cag* pathogenicity island as a determinant of gastric cancer risk

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ABSTRACT

Helicobacter pylori strains can be broadly classified into two groups based on whether they contain or lack a chromosomal region known as the *cag* pathogenicity island (*cag* PAI). Colonization of the human stomach with *cag* PAI-positive strains is associated with an increased risk of gastric cancer and peptic ulcer disease, compared to colonization with *cag* PAI-negative strains. The *cag* PAI encodes a secreted effector protein (CagA) and components of a type IV secretion system (Cag T4SS) that delivers CagA and non-protein substrates into host cells. Animal model experiments indicate that CagA and the Cag T4SS stimulate a gastric mucosal inflammatory response and contribute to the development of gastric cancer. In this review, we discuss recent studies defining structural and functional features of CagA and the Cag T4SS and mechanisms by which *H. pylori* strains containing the *cag* PAI promote the development of gastric cancer and peptic ulcer disease.

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H. pylori cag pathogenicity island

Helicobacter pylori are Gram-negative bacteria highly adapted for colonization of the human stomach. About half of the world's population is persistently colonized by these bacteria.[1] Most individuals never develop adverse consequences attributable to *H. pylori* colonization, but the presence of *H. pylori* confers an increased risk of peptic ulcer disease and gastric cancer.[2,3]

H. pylori strains isolated from unrelated individuals exhibit a high level of genetic diversity.[4-6] One of the most striking differences among *H. pylori* strains is the presence or absence of a 40-kb chromosomal region known as the *cytotoxin-associated gene* pathogenicity island (*cag* PAI).[7,8] The *cag* PAI has a guanine-cytosine content substantially lower than the rest of the *H. pylori* chromosome, which suggests that it was acquired through a horizontal transfer event. Since *cag* PAI-positive strains are geographically dispersed in human populations throughout the world, the *cag* PAI was probably acquired by an ancestral strain prior to human migrations out of Africa.[9]

In most *cag* PAI-positive *H. pylori* strains, the entire 40-kb *cag* PAI is localized between a Sel1-like gene (HP0519 in prototype strain 26695) and a gene encoding glutamate racemase (*glr*, corresponding to HP0549 in strain 26695). The gene content and gene order within the *cag* PAI are relatively well-conserved among unrelated *H. pylori* strains[9] (Figure 1). Variations can result from gene deletions, gene insertions (sometimes associated with IS605 or IS606 elements), genomic rearrangements, or gene inversions. In some strains, fragments of the *cag* PAI are distributed in separate chromosomal loci.[7,8]

Early studies noted that one gene within the *cag* PAI encodes an immunodominant antigenic protein (CagA) recognized by human serum antibodies.[12,13] Subsequent studies showed that CagA is a secreted effector protein delivered into host cells by a type IV secretion system (Cag T4SS), components of which are encoded by genes within the *cag* PAI.[14-19] The Cag T4SS also delivers several types of non-protein substrates into host cells (Figure 2).[20] Epidemiologic studies, coupled with

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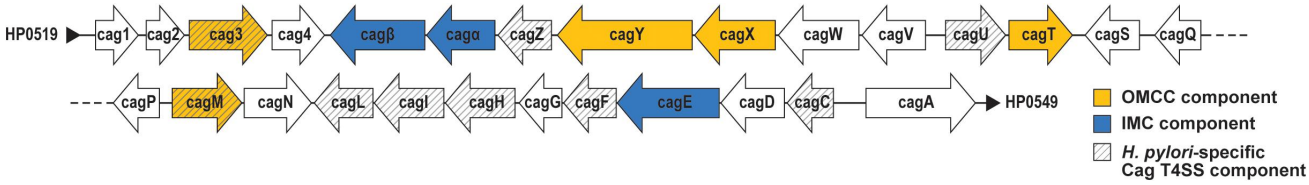

Figure 1. Organization of genes within the *cag* PAI. Five genes encoding proteins localized to the T4SS OMCC[10,11] and three genes encoding putative ATPases localized to the T4SS IMC are indicated. Genes required for Cag T4SS activity that lack homologs in other bacterial species are indicated with diagonal stripes.

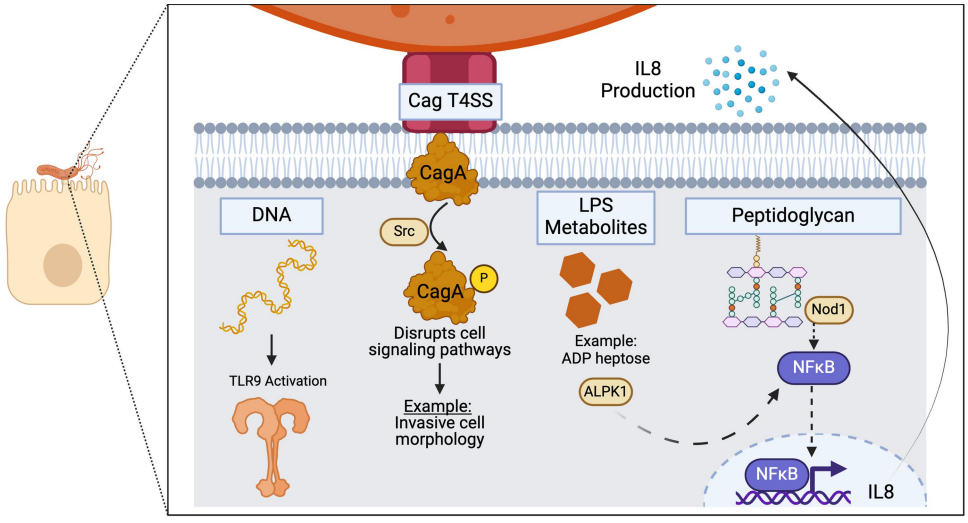

Figure 2. Cag T4SS-mediated delivery of CagA and non-protein substrates into host cells. The Cag T4SS is required for delivery of CagA, LPS metabolites, peptidoglycan and DNA into host cells. Each substrate elicits a cellular response. CagA is phosphorylated by tyrosine kinases (such as Src), and phosphorylated CagA can cause disruptions to a variety of signaling pathways. LPS metabolites and peptidoglycan elicit NF-κB activation, leading to IL-8 production. DNA translocation causes TLR9 activation. Created with BioRender.com.

cell biology and animal model experiments, have demonstrated important roles of CagA and the Cag T4SS in the pathogenesis of both gastric cancer and peptic ulcer disease, and recent studies have provided important insights into functional and structural properties of CagA and the Cag T4SS. In this review, we provide an overview of CagA and the Cag T4SS, and we discuss mechanisms by which products of the *cag* PAI contribute to the pathogenesis of gastric cancer and peptic ulcer disease.

Epidemiologic links between *cag* PAI-positive *H. pylori* strains and gastric disease

Early serologic studies noted that serum antibodies or gastric mucosal IgA antibodies to CagA were detected more commonly in individuals with peptic ulcer disease than in *H. pylori*-positive control patients without ulcers or *H. pylori*-negative individuals.[12,13,21–24] Serum antibodies to CagA are also detected more commonly in individuals with gastric adenocarcinoma or gastric premalignant conditions (such as atrophic gastritis or intestinal metaplasia) than in *H. pylori*-positive control patients or *H. pylori*-negative individuals.[25-34]

The presence of anti-CagA serum antibodies is correlated with the presence of *cagA*-positive *H. pylori* strains in the stomach.[23] Accordingly, genetic analyses of *H. pylori* strains or gastric tissue samples have shown that *cagA*-positive strains are detected more commonly in individuals with gastric adenocarcinoma or premalignant lesions than in *H. pylori*-positive individuals with non-atrophic gastritis only.[35-46] In a large study of 2145 patients from Venezuela, there was a strong association between the presence of *cagA*-positive strains and premalignant gastric lesions.[36] Specifically, the odds ratio for gastric dysplasia (a statistical assessment of relative

risk) was 15.5 (95% confidence interval 6.42 to 37.2) in individuals colonized with *cagA*-positive strains compared with *H. pylori*-negative individuals, and 0.90 (95% confidence interval 0.37 to 2.17) in individuals colonized with *cagA*-negative strains compared with *H. pylori*-negative individuals. The proportion of *cagA*-positive *H. pylori* strains is also higher among individuals with duodenal or gastric ulcers than among individuals with non-atrophic gastritis,[37,41,47-50] especially if cases of ulcers caused by non-steroidal anti-inflammatory drugs are excluded. Similarly, the severity of gastric inflammation is typically higher among individuals colonized with *cagA*-positive strains than among those colonized with *cagA*-negative strains.[40,51,52]

The detection of *cagA* in *H. pylori* strains or gastric samples suggests that the *cag* PAI is present, but further analyses of *H. pylori* strains, such as whole genome sequencing or functional assays to assess T4SS-dependent phenotypes, are required to verify the presence of an intact *cag* PAI. Genetic detection of an "empty site locus" in the *H. pylori* chromosomal region between HP0519 (*sel1*-like gene) and HP0549 (*glr*) can provide evidence that the *cag* PAI is absent.[53] Most analyses of *H. pylori* strains or clinical samples have assessed the presence or absence of *cagA* in relation to gastric disease states, without assessing whether an intact *cag* PAI is present. Notably, a genome-wide association study of 173 *H. pylori* isolates from European patients with defined disease strains demonstrated an association between multiple genes in the *cag* PAI and gastric cancer.[54]

Isolation of *H. pylori* strains from the stomach for genetic analysis or detection of *cag* PAI genes in gastric specimens typically requires sampling of the stomach using endoscopic biopsies, which evaluate only small portions of the stomach. Therefore, CagA serologic tests are potentially more sensitive methods for detecting *cagA*-positive *H. pylori* strains, compared to genetic methods. The detection of anti-CagA antibodies can reflect either active *H. pylori* colonization or previous colonization.[30]

Most studies analyzing relationships between gastric disease states and CagA (or the *cag* PAI) have been cross-sectional or case-control analyses, comparing groups of symptomatic patients with different disease states who underwent upper gastrointestinal endoscopy. Further insights have come from studies of serum samples collected decades prior to the development of gastric disease. Analysis of the stored serum samples demonstrated an association between CagA seropositivity and the subsequent development of gastric cancer.[31] Additional insights have come from the analysis of serial gastric biopsies collected over time. In a large prospective study of Venezuelan patients (mean follow-up 3.5 years), gastric biopsies were analyzed to detect progression or regression of premalignant lesions.[36] Individuals colonized with *cagA*-positive *H. pylori* strains were more likely to exhibit progression of premalignant lesions than those colonized with *cagA*-negative strains, but the differences were not statistically significant. Similarly, a longitudinal study of patients in Spain showed that colonization with *cagA*-positive strains was associated with progression of preneoplastic lesions.[44]

Most studies demonstrating a positive correlation between CagA or the *cag* PAI and disease states have been conducted in geographic regions where both *cagA*-positive and *cagA*-negative *H. pylori* strains are commonly isolated. Such relationships have been less frequently detected in East Asia or other geographic regions where nearly all *H. pylori* isolates are *cagA*-positive.[55,56]

Individuals with a history of duodenal ulcer disease have a reduced incidence of gastric cancer compared to matched control patients without a history of duodenal ulceration.[57] Therefore, the association of *cag* PAI-positive strains with an increased risk of both gastric cancer and duodenal ulcer disease is somewhat surprising. One possible explanation is that the *cag* PAI contributes to the pathogenesis of both diseases (for example, by stimulating gastric mucosal inflammation), and host-specific traits related to levels of gastric acid production determine whether an individual is predisposed to develop duodenal ulceration or gastric cancer.

Properties of the CagA effector protein

CagA is an immunodominant *H. pylori* protein that was originally identified based on its antigenic

properties.[12,13,21,22] CagA is recognized by both human serum antibodies and gastric mucosal IgA antibodies.[12,13,21,22] The molecular masses of CagA proteins produced by unrelated *H. pylori* strains range from about 120 kDa to 150 kDa. The sequence of CagA does not exhibit relatedness to sequences of proteins in other bacterial species. The structure of the amino-terminal portion of CagA (residues 1–829) has been determined by X-ray crystallography,[58,59] and three structurally distinguishable domains within this portion of CagA have been described. The relatively unstructured carboxy-terminal portion of CagA contains motifs important for CagA activity and a C-terminal secretion signal, discussed further in subsequent sections.

The first evidence for CagA entry into host cells came from experiments in which gastric epithelial cells were co-cultured with *cagA*-positive *H. pylori* strains. A tyrosine-phosphorylated ~130 kDa band was detected in lysates of the co-culture mixtures but not in lysates of uninfected gastric cells, and this band was shown to be a tyrosine-phosphorylated form of CagA.[14-19] Subsequent studies have detected CagA entry into host cells using a translocation reporter assay in which β-lactamase (TEM-1) is fused to CagA,[60] or by use of a split luciferase (HiBiT) translocation reporter assay.[61] Upon delivery into gastric cells, CagA localizes to the inner leaflet of the plasma membrane in a multimeric state.[62,63]

Tyrosine phosphorylation of CagA within host cells occurs on tyrosine residues within CagA EPIYA motifs (glutamate-proline-isoleucine-tyrosine-alanine), located within the C-terminal unstructured portion of CagA,[64,65] and is mediated by tyrosine kinases (including c-Src, Fyn, Lyn, YES, and Abl).[66,67] Tyrosine-phosphorylated CagA can interact with a large number of intracellular proteins, resulting in alterations of protein function.[68-70] Non-phosphorylated CagA can also interact with host cell proteins and alter their activity.[71-75] The interactions involving non-phosphorylated CagA are mediated by one or more sites designated "conserved repeat responsible for phosphorylation-independent activity" (CRPIA) motifs, located within the C-terminal unstructured portion of CagA.[73]

Unrelated *H. pylori* strains contain variable numbers and types of CagA EPIYA motifs. Different EPIYA motifs are selectively phosphorylated by different kinases in a stepwise process; therefore, CagA is phosphorylated on only one or two EPIYA motifs.[76] Variation among *H. pylori* strains in the types of EPIYA motif sequences, the number of copies of EPIYA motifs, and the arrangement of the motifs contributes to variation in CagA activity among strains in vitro.[77,78] Moreover, differences among strains in the number and type of EPIYA motifs have been correlated with differences in gastric disease risk.[79] Geographic variations in CagA EPIYA motifs and the associated impacts on CagA activity and gastric disease are discussed further in a subsequent section.

Properties of the Cag T4SS

At least 16 genes within the *H. pylori cag* PAI are required for delivery of CagA into host cells.[19,20,80] Several of these genes encode proteins exhibiting sequence relatedness to the components of T4SSs in other bacterial species. T4SSs are a versatile group of nanomachines that can transport an assortment of substrates, including protein and DNA.[81–84] T4SSs are widespread among both Gram-negative and Gram-positive bacterial species and are also present in Archaea. Two of the most common actions of T4SSs are horizontal transfer of DNA among bacteria (conjugation) and delivery of effector proteins into target cells.[81-84]

Prototype T4SSs in Gram-negative bacteria (conjugation systems and the *Agrobacterium tumefaciens* VirB/VirD4 system) are composed of 12 protein components, designated VirB1–11 and VirD4.[81-84] Most of these components are organized into two large subassemblies known as the outer membrane core complex (OMCC) and inner membrane complex (IMC).[84-86] The OMCC is localized within the periplasm and includes proteins that interact with the outer membrane and/or inner membrane. The IMC spans the inner membrane and includes proteins projecting into the cytoplasm and periplasm. Three protein components (VirB7, VirB9, and VirB10) compose the OMCC in prototype T4SSs. The IMC is composed of three ATPases (VirB4, VirB11, and VirD4), along with VirB3 and VirB8. Additional proteins (VirB5 and VirB6) localize to a stalk connecting the OMCC and IMC.[86] An extracellular pilus structure (composed of two protein

components, VirB2, and VirB5) is present in T4SSs from some bacterial species.[84]

The overall structural organization of the *H. pylori* Cag T4SS has multiple features resembling those of prototype T4SSs, including the presence of an OMCC and IMC[85] (Figure 3). Cryo-electron tomography (cryo-ET) analyses of intact *H. pylori* has allowed visualization of these Cag T4SS subassemblies in situ (in intact bacteria).[87,88] The Cag T4SS OMCC is a large mushroom-shaped complex localized between the inner membrane and the outer membrane. The portion of the OMCC visualized by cryo-ET has 14-fold symmetry.[87,88] The IMC has 6-fold symmetry and consists of three concentric rings surrounding a central channel.[88] The OMCC and IMC are connected by a stalk region. Other Cag T4SS-associated features visualized by cryo-ET (with low resolution) include periplasmic elements designated as "wings" or a "collar".[87,88] These probably correspond to regions of prototype T4SSs designated as "arches".[86]

The *H. pylori* Cag T4SS OMCC remains intact in the presence of detergent, which has facilitated isolation and detailed structural analysis of this subassembly, using single-particle cryo-electron microscopy (cryo-EM).[10,11,89] The OMCC is about 41 nm in diameter and is composed of three main subassemblies: an outer membrane cap (OMC), a periplasmic ring (PR) and a stalk. The OMC has 14-fold symmetry and contains 5 proteins (CagY, CagX, CagT, CagM, and Cag3) in a 1:1:2:2:5 stoichiometric ratio.[10,11] In total, the OMC portion of the OMCC contains 154 polypeptide chains.[11] The periplasmic ring (PR) has 17-fold symmetry and contains only CagX and CagY.[11] CagY, CagX, and CagT are homologous to VirB10, VirB9 and VirB7 components of T4SSs in other bacterial species, whereas CagM and Cag3 are species-specific components of the Cag T4SS. CagY (like VirB10 components in other bacterial T4SSs) is predicted to span from the outer membrane to the inner membrane,[90] but thus far, only the C-terminal portion of CagY has been structurally defined. Both cryo-ET analysis of intact *H. pylori* and cryo-EM analysis of isolated OMCCs have detected a stalk-like structure that connects the OMCC with the IMC,[10,88] but the molecular composition of the stalk has not yet been defined.

A high-resolution structural model is not yet available for the Cag T4SS IMC, but insights into its composition have been provided by cryo-ET analysis of the T4SS in intact bacteria,[88] combined with comparisons to the structural organization of the IMC of a conjugation system.[86] Similar to

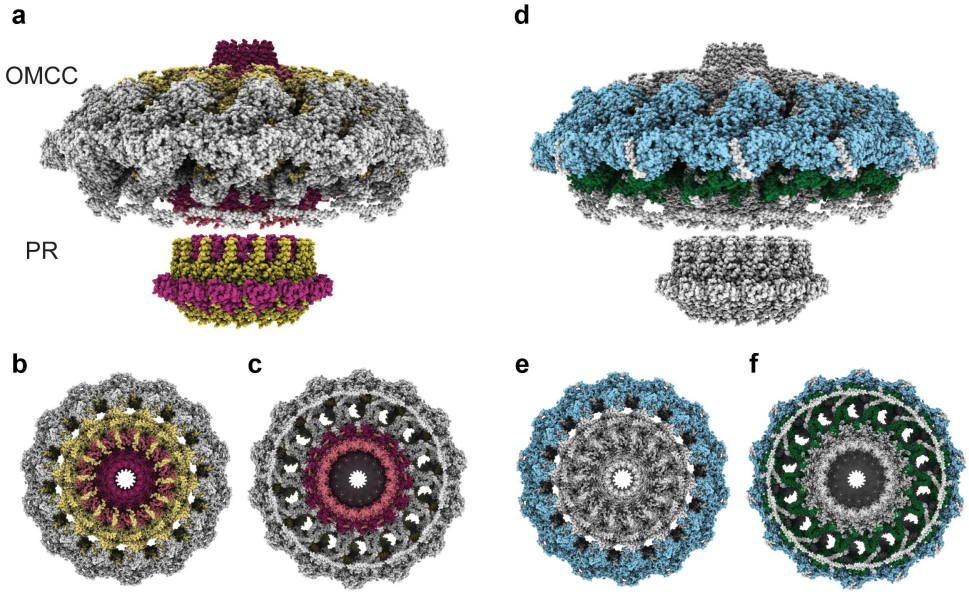

Figure 3. Structural organization of the Cag T4SS OMCC. The 14-fold-symmetric OMC and 17-fold-symmetric PR are illustrated.[10,11] (a, b, c) conserved Cag T4SS components. Purple = CagY, yellow = CagX, pink = CagT. (d, e, f) *H. pylori*-specific T4SS components. Blue = Cag3, green = CagM.

prototypical T4SSs, the Cag T4SS IMC is predicted to contain three putative ATPases (Cagα, Cagβ, CagE), corresponding to VirB11, VirD4, and VirB3/VirB4, respectively. Each of these putative ATPases is required for CagA secretion.[19,91] Cryo-ET analysis of *H. pylori* mutant strains with deletions of genes encoding individual ATPases revealed that CagE constitutes the IMC density closest to the inner membrane, followed by Cagα, and then Cagβ, which makes up the cytoplasmic density furthest from the inner membrane.[88] The Cag T4SS IMC contains additional densities that were unable to be assigned to the three ATPases. These might correspond to CagV (a VirB8 homolog) and CagU, which are predicted components of the IMC.

Among the *cag* PAI-encoded proteins required for CagA translocation, five are components of the OMCC[10,11] and at least five are known or predicted to be components of the IMC[88] (Table 1). The localization of several species-specific Cag proteins required for CagA translocation (lacking sequence relatedness to T4SS components in prototype systems) remains unclear. CagF is proposed to be a cytoplasmic protein, functioning as a CagA chaperone, but it might also be associated with the inner membrane.[92-94] CagW (a VirB6 homolog) might be a component of the stalk, based on the observed localization of VirB6 to the stalk in a conjugation system.[86] CagH, CagI, and CagL physically interact to form a complex,[95,96] but the subcellular localization of these proteins remains unclear. CagC, CagL, and CagI have been detected on the surface of *H. pylori*,[97-99] and it has been proposed that these proteins might interact with receptors on host cells.

T4SSs in some bacterial species include extracellular pilus structures, composed of VirB2 and VirB5 components.[84] It has been proposed that *H. pylori* CagC and CagL might be VirB2- and VirB5-like components, respectively,[98,100] but there is no sequence or structural relatedness between *H. pylori* CagL and VirB5 proteins from other bacterial species. Several papers have reported the production of extracellular pilus-like structures by *H. pylori*,[97,101] but the relationship between these structures and the Cag T4SS remains unclear. In a cryo-ET analysis of *H. pylori* co-cultured with AGS gastric cells, the pilus-like structures were described as "membranous tubes with lateral ports".[87] These pilus- or tube-like structures were never visualized in association with the Cag T4SS OMCC, and similar outer membrane protrusions, unrelated to T4SSs, have been visualized in many bacterial species.[102]

In comparison to prototype T4SSs (classified as minimized T4SSs), the Cag T4SS is much larger in size. For example, the diameter of the Cag T4SS

Table 1. *cag* PAI-encoded proteins that contribute to Cag T4SS activity.

Protein name	Synonym	Homologs	Molecular mass (kDa)	Localization or predicted localization [a]	Putative function
CagY	Cag7	VirB10	219	OMCC	
CagX	Cag8	VirB9	61	OMCC	
CagT	Cag12	VirB7	32	OMCC	
Cagα		VirB11	37	IMC	ATPase
Cagβ	Cag5	VirD4	86	IMC	ATPase
CagE	Cag23	VirB3/ VirB4	112	IMC	ATPase
CagC	Cag25	VirB2	12	IM, OM, S	
CagV	Cag10	VirB8	29	IM	
Cag4		VirB1	20	PP	PG hydrolase
CagA	Cag26		132	C, S	Effector protein
CagD	Cag24		24	IM, PP, S	
CagF	Cag22		32	IM, C	CagA chaperone
CagG	Cag21		16	PP	
CagH	Cag20		39	IM	
CagI	Cag19		42	PP	
CagL	Cag18		27	PP, S	
CagN	Cag17		35	PP, IM	
CagM	Cag16		44	OMCC	
CagU	Cag11		25	IM	
CagW	Cag9	VirB6	58	IM	
CagZ	Cag6		23	IM	
Cag3			55	OMCC	

[a]Protein localizations are based on subcellular fractionation analysis, cryo-ET analysis of the T4SS in intact bacteria, or single particle cryo-EM analysis of isolated complexes. Predicted localizations are based on based on protein sequence analysis or fractionation experiments. OM, outer membrane; IM, inner membrane; C, cytoplasm; S, surface-exposed or supernatant; PP, periplasm.

OMCC (approximately 41 nm) is nearly double the diameters of OMCCs in prototypical T4SSs.[85] Among the *cag* PAI genes required for delivery of CagA into host cells, 10 (CagY, CagX, CagT, Cagα, Cagβ, CagE, CagV, CagW, Cag4, and CagC) exhibit relatedness to VirB/VirD4 proteins in prototype systems, and 8 (Cag3, CagM, CagH, CagI, CagL, CagF, CagU, and CagZ) are found uniquely in *H. pylori* (Table 1). Therefore, the *H. pylori* Cag T4SS has been classified as an "expanded T4SS", along with the *Legionella pneumophila* Dot/Icm T4SS and the *Coxiella burnetii* T4SS.[82,84,85]

In addition to the classification of T4SSs into two groups (minimized and expanded) based on the number of components and physical size of the membrane-spanning structures, T4SSs have been classified into two groups (type IVA and type IVB) based on phylogenetic analysis of conserved components.[103,104] Most type IVA systems, including the prototypical *Agrobacterium tumefaciens* VirB/VirD4 system, contain the minimum 12 proteins required for T4SS function, whereas type IVB systems, such as the *Legionella* Dot/Icm T4SS and the *Coxiella* T4SS, contain multiple additional species-specific components. Despite having limited sequence homology to the VirB/VirD4 system and containing multiple species-specific components, the *H. pylori* Cag T4SS has been classified as a type IVA secretion system.[103]

Cag T4SS-mediated delivery of CagA into host cells

H. pylori contact with gastric epithelial cells triggers CagA secretion and delivery into host cells. In contrast, CagA is not secreted into the extracellular milieu if *H. pylori* is cultured in vitro under routine conditions. The stimuli that trigger CagA secretion have not yet been defined. The carboxy-terminal portion of *H. pylori* CagA contains a 20-amino-acid secretion signal, similar to secretion signals found in T4SS effector proteins in other bacterial species.[105] Both the carboxy-terminal motif and a large ~ 350-amino-acid segment within the amino-terminal portion of CagA (Domains I and II) are required for CagA secretion.[94] Current evidence suggests that CagA is secreted in an unfolded state. Specifically, a CagA-dihydrofolate reductase (DHFR) fusion protein is unable to be translocated when DHFR folding is stabilized with methotrexate.[61] Similarly, a CagA-GFP fusion protein is not delivered into host cells and can exert a dominant-negative inhibitory effect on secretion of wild-type CagA.[105]

CagA physically interacts with CagF, a *H. pylori*-specific protein proposed to be a cytoplasmic chaperone that stabilizes CagA prior to its recruitment to the T4SS apparatus.[92-94] Cagβ (a VirD4 ATPase homolog) is predicted to be required for CagA recruitment to the T4SS apparatus, based on the role of VirD4 as a coupling protein in prototypical T4SSs. Cagβ interacts with a species-specific Cag protein known as CagZ.[106] It has been proposed that Cagβ does not incorporate into the IMC when bound to CagZ.[106] The stimuli promoting CagA binding or release from CagF, CagZ binding or release from Cagβ, and CagA recruitment to the T4SS apparatus have not yet been defined.

The precise mechanisms by which CagA is transported through the T4SS apparatus are not known. Yeast two-hybrid experiments and other in vitro analyses suggest that CagA can interact not only with CagF and Cagβ, but also with multiple additional T4SS components.[107,108] In addition to Cagβ, two other putative ATPases (Cagα and CagE) are required for CagA delivery into host cells.[19,91] The specific secretory steps powered by these individual ATPases are not known.

Interactions between the Cag T4SS and the surface of host cells are poorly understood. Several Cag proteins (including CagC, which exhibits weak sequence relatedness to VirB2 pilins)[98] are reported to be present on the *H. pylori* surface, and these can potentially interact with host cells. CagL, CagI, and CagY have been reported to interact with various integrins.[97,109,110] These interactions are presumed to be important in mediating interactions between the T4SS and host cells. Integrins are localized to the basolateral surface of gastric epithelial cells and are predicted to be inaccessible to *H. pylori* bound to the apical surface of gastric epithelial cells.[111] Experiments with polarized epithelial monolayers indicate that the activity of *H. pylori* proteases such as HtrA promotes *H. pylori* access to the basolateral surface of epithelial cells, thereby facilitating interactions of Cag T4SS components with integrins.[111,112]

The mechanisms by which CagA is translocated across the plasma membrane of host cells also remain poorly understood. Thus far, there is not any evidence indicating that Cag T4SS components directly insert into the plasma cell of host cells (analogous to the translocon of type III secretion systems). Therefore, CagA might enter host cells through endocytic processes. CagA can physically interact with β1 integrin,[59,109] which provides a potential route for CagA binding and entry into host cells. As an additional or alternate route for CagA entry into host cells, *H. pylori* contact with epithelial cells induces externalization of phosphatidylserine to the outer leaflet of the plasma membrane, and CagA can interact with phosphatidylserine.[113]

Intracellular actions of CagA

Early studies noted that the entry of CagA into gastric epithelial cells was associated with an alteration of cellular morphology known as the "hummingbird phenotype", characterized by an elongation of cell shape and cytoskeletal alterations.[14] Subsequent studies showed that CagA causes numerous additional alterations in host cells, including loss of cell polarity,[114] increased cell motility,[75,115,116] cell scattering,[71,115,117] cell proliferation,[71,73] cell invasiveness,[114] an epithelial to mesenchymal transition-like phenotype,[114] disruption of intercellular junctions, and disruption of epithelial barrier functions.[118] These cellular changes are the consequences of CagA interactions with multiple intracellular proteins. CagA (in either phosphorylated or non-phosphorylated forms) is reported to interact with at least a dozen proteins in host cells, leading to cellular alterations that are relevant for oncogenesis (discussed in previous reviews).[68,69] Several examples are presented here.

One of the most extensively studied CagA interactions is the binding of tyrosine-phosphorylated CagA to Src homology region 2 (SH2)-containing protein tyrosine phosphatase 2 (SHP2).[119] Under physiologic conditions, SHP2 is in an enzymatically inactive conformation. The interaction of phosphorylated CagA EPIYA motifs with SHP2 triggers a conformational change, resulting in an active form of SHP2 that stimulates signaling pathways involved in cell morphology, motility, and proliferation.[68] Phosphorylated CagA interacts with Src-homology 2 domains in several additional proteins, including C-terminal SRC kinase (CSK).[120] CagA interactions with SHP2 contribute to the "hummingbird phenotype" observed following *H. pylori* co-culture with gastric epithelial cells.[119]

Another extensively studied CagA interaction is its binding to the partitioning defective 1 (PAR1) family of serine/threonine kinases [known as microtubule affinity-regulating kinases (MARKs)], which are important in maintaining the polarization of epithelial cells and tight junctions, as well as microtubule dynamics.[69,74,121] CagA interactions with PAR1/MARK proteins require the CagA multimerization (CM) motif but are not dependent on the phosphorylation state of CagA.[69,74,121] Inhibition of PAR1 function by CagA contributes to the hummingbird phenotype[122] and results in mislocalization of tight junction proteins (e.g., ZO-1) and basolateral proteins (such as E-cadherin), leading to defects in cell polarity and impaired tight junction barriers.[74]

CagA also interacts with E-cadherin (a component of adherens junctions).[123,124] This interaction results in the destabilization of the E-cadherin/β-catenin complex, translocation of β-catenin to the nucleus,[125] and the activation of Wnt signaling. CagA may also activate Wnt signaling through additional mechanisms.[68,126]

CagA interacts with apoptosis-stimulating protein of p53 (ASPP2),[127] which promotes the proteasomal degradation of the tumor suppressor p53.[128] The interaction of CagA with ASPP2 leads to inhibition of apoptosis (resistance to cell death). CagA can also negatively regulate p53 through additional mechanisms.[129,130]

Another important consequence of CagA intracellular activity is stimulation of DNA damage and double-strand DNA breaks in host cells.[131-133] One mechanism involves inactivation of PAR1b, leading to alteration of PAR1b-dependent BRCA1 phosphorylation and impaired nuclear localization of BRCA1.[134] Another mechanism involves upregulation of spermine oxidase and resulting oxidative stress.[131] CagA is also implicated in the downregulation of several genes involved in DNA repair.[133,135]

Cag T4SS-mediated delivery of non-protein substrates into host cells

Early studies noted that co-culture of *cag* PAI-positive *H. pylori* strains with gastric epithelial cells stimulated the production and secretion of interleukin-8 (IL-8), a proinflammatory cytokine that promotes recruitment and activation of neutrophils.[7,8,19,136] This phenotype was dependent on multiple *cag* PAI genes encoding Cag T4SS components but did not require CagA.[7,8,19,136] The IL-8 phenotype, a consequence of NF-κB activation, is now known to result primarily from the entry of non-protein *H. pylori* substrates into host cells. Although CagA is not required for IL-8 induction,[19] CagA can contribute to the capacity of some *H. pylori* strains to stimulate IL-8 production in gastric epithelial cells.[137]

Mutagenesis of several *H. pylori* genes required for LPS inner core heptose biosynthesis (*gmhA*, *hldE*, and *rfaE*) leads to a marked reduction in the capacity of *H. pylori* to stimulate NF-κB activation and IL-8 production.[138,139] In contrast, these mutations do not inhibit T4SS-mediated delivery of CagA into host cells. These findings suggested that *H. pylori* LPS intermediates might be mediators of the IL-8 phenotype. Initial studies concluded that *H. pylori* heptose 1,7-bisphosphate (HBP) was the relevant pathogen-associated molecular pattern (PAMP),[138-140] similar to what had been reported previously in studies of other bacterial species.[141-143] Subsequent studies found that *H. pylori* lysates contain very low concentrations of heptose 1,7-bisphosphate (HBP) and identified ADP-glycero-β-D-manno-heptose (ADP heptose), a derivative of HBP, as a more active *H. pylori* PAMP.[144] Cag T4SS-dependent activation of NF-κB and IL-8 production by LPS metabolites is a consequence of the activation of alpha kinase 1 (ALPK1), which activates the TRAF-interacting protein with forkhead domain (TIFA).[139,145] TIFA then forms large complexes (TIFAsomes) composed of TIFA and other cellular proteins, including TRAF2, leading to the activation of NF-κB.[145]

Co-culture of *H. pylori* with gastric epithelial cells results in activation of Nod1 through a Cag T4SS-dependent process, which provides an additional mechanism for NF-κB activation and IL-8 production.[146] Peptidoglycan from many bacterial species is known to be a stimulus for Nod1 activation. In the case of *H. pylori*, meso-diaminopimelate (mDAP)-containing N-acetylglucosamine-N-acetylmuramic acid (GM-tripeptide) is the peptidoglycan moiety that is specifically recognized by Nod1. The results of one study suggested that Nod1 activation has a minimal role in IL-8 activation compared to the activation of the TIFA pathway.[139]

Another consequence of *H. pylori* co-culture with gastric epithelial cells is the activation of Toll-like receptor 9 (TLR9).[147] This phenotype is dependent on Cag T4SS activity and is attributed to the entry of *H. pylori* DNA into host cells.[147,148] TLR9 receptors recognize unmethylated CpG motifs on DNA, which are predominantly found on bacterial or viral DNA but not mammalian DNA. Activation of TLR9 may lead to a dampening of the inflammatory response by suppressing IL-17-mediated responses.[149] The anti-inflammatory effect of TLR9 activation contrasts with the pro-inflammatory effects resulting from entry of LPS metabolites and peptidoglycan into host cells and might promote persistent *H. pylori* colonization.

Relatively little is known about the T4SS-dependent processes by which non-protein substrates are delivered into host cells. In contrast to CagA secretion, which requires three ATPases (CagE, Cagα, and Cagβ), delivery of non-protein substrates into host cells requires CagE and Cagα but not Cagβ.[19,91] Similarly, CagF is required for CagA secretion but not delivery of non-protein substrates.[92,93]

To systematically identify *H. pylori* genes required for T4SS-dependent processes, one study screened a *H. pylori* transposon mutant library to identify mutants unable to activate NF-κB in gastric epithelial cells.[150] As expected, this analysis identified numerous *cag* PAI genes and also identified three non-*cag* PAI genes: *hopQ* (which encodes an outer membrane protein), a gene encoding a predicted LPS glycosyltransferase (HP0159), and a gene encoding a predicted flagellar-associated protein (HP1029/1028). Subsequent studies showed that HopQ is an outer membrane protein that interacts with carcinoembryonic

antigen-related cell adhesion molecules (CEACAMs) on host cells, thereby promoting T4SS-mediated delivery of substrates into host cells.[151-153]

Two additional *H. pylori* outer membrane proteins (BabA and AlpA/B) are reported to contribute to Cag T4SS-dependent processes.[154,155] Other non-*cag* PAI genes reported to contribute to T4SS-dependent processes include *hyd* (encoding hydrogenase) and HP1564 (encoding a protein of unknown function).[156,157]

In addition to cellular responses resulting from T4SS-dependent entry of CagA and non-protein substrates into host cells, there is evidence that components of the T4SS can directly cause cellular responses.[158] One mechanism involves interactions of Cag proteins with Toll-like receptor 5 (TLR5), a receptor that typically recognizes bacterial flagellins. *H. pylori* flagellin is adapted to avoid recognition by TLR5,[159,160] but two T4SS components (CagL and CagY) can directly interact with TLR5 and activate this receptor, leading to downstream signaling that triggers the production of specific cytokines and chemokines.[161,162]

In summary, CagA is the only protein known to be secreted and translocated by the Cag T4SS. The Cag T4SS can also deliver multiple types of non-protein substrates into host cells, resulting in proinflammatory signaling (ADP-heptose and peptidoglycan) or anti-inflammatory signaling (DNA). Unusual features of the Cag T4SS include its capacity to trigger cellular alterations through the properties of T4SS components, independent of the translocation of the effector molecule.

Activities of CagA and the T4SS in animal models

Several transgenic animal models have been used to evaluate the consequences of intracellular CagA activity in vivo. Transgenic mice engineered to express CagA developed gastric epithelial hyperplasia, gastric polyps, adenocarcinomas of the stomach and small intestine, myeloid leukemias, and B cell lymphomas.[163] Similarly, transgenic zebrafish expressing CagA developed hyperplasia of the adult intestinal epithelium.[164] Intestinal hyperplasia was detected in zebrafish following long-term transgenic expression of wild-type CagA, but not a phosphorylation-resistant form of CagA.[164] Transgenic expression of CagA in a *Drosophila* model resulted in an assortment of abnormalities in morphogenesis, including alterations in ocular photoreceptor development.[165,166] Expression of CagA within *Drosophila* intestinal stem cells promoted excess cell proliferation and led to alterations in host microbiota.[167] Ectopic expression of CagA in *Xenopus laevis* embryos resulted in impaired gastrulation, neural tube formation, and axis elongation.[126] Therefore, transgenic CagA expression in vivo results in extensive cellular alterations and oncogenic effects, consistent with studies of CagA action in vitro. Notably, transgenic expression of CagA in mice did not lead to a prominent inflammatory response.[163]

Experimental intragastric administration of *H. pylori* to mice results in *H. pylori* colonization of the stomach and detectable gastric inflammation, but gastric ulceration and gastric cancer do not develop in wild-type mice infected with *H. pylori*. *H. pylori* colonization of the mouse stomach does not require the *cag* PAI, and during colonization of the mouse stomach, *cag* PAI-positive *H. pylori* strains commonly acquire mutations leading to inactivation of Cag T4SS function.[168] Moreover, mouse gastric epithelial cells are resistant to the actions of CagA due to the inability of HopQ to bind to mouse CEACAMs.[169] The loss of Cag T4SS activity in vivo, combined with resistance of mouse cells to CagA activity, may account at least in part for the absence of gastric cancer or gastric ulceration in *H. pylori*-infected wild-type mice.

The apparent selective advantage of strains lacking Cag T4SS function in mice complicates efforts to study a potential contribution of the *cag* PAI to gastric inflammation or gastric disease in wild-type mouse models. Nevertheless, several studies have reported that CagA or the T4SS contribute to gastric inflammation and gastric disease in wild-type mice.[170,171] Two studies have shown that the activation of gastric stem cell populations (Lgr5- or Lrig-1-positive cells) is dependent on Cag T4SS activity.[171,172] In a transgenic hypergastrinemic mouse model of gastric carcinogenesis (INS-GAS), there was a trend toward delayed development of

gastric cancer in animals infected with a *cagE* mutant strain compared to a wild-type strain.[173]

Administration of *cag* PAI-positive *H. pylori* to Mongolian gerbils commonly results in severe gastric inflammation, often accompanied by gastric ulceration, premalignant changes, and gastric adenocarcinoma.[174-183] In contrast, *cagA* mutant strains and mutant strains defective in Cag T4SS activity cause only mild gastric inflammation and do not cause ulceration or gastric cancer in the gerbil model.[174-181,183] Similarly, an *H. pylori* strain in which expression of Cag T4SS components is controlled by the TetR/*tetO* system caused more severe gastric inflammation and disease under conditions in which the expression of relevant genes (*cagU* and *cagT*) was de-repressed than under conditions in which expression was repressed.[184] One study reported that the *H. pylori* colonization density was higher in gerbil stomachs colonized with wild-type *H. pylori* strains than in gerbil stomachs colonized with *cagA* mutants.[177]

Non-human primate models have also been used to assess the effects of *H. pylori* CagA and the Cag T4SS in vivo. Rhesus macaques experimentally infected with a wild-type *H. pylori* strain developed increased gastric mucosal inflammation compared to animals infected with a *cag* PAI mutant strain.[185]

Geographic variations in prevalence of *cag* PAI-positive strains and features of CagA EPIYA motifs

Estimates of the prevalence of *cag* PAI-positive *H. pylori* strains within populations have been based on analysis of symptomatic patients who underwent endoscopic procedures because of gastric symptoms. The results of such studies may not accurately reflect the prevalence of *cag* PAI-positive strains within asymptomatic populations. Nevertheless, the available data indicate that there are geographic variations in the prevalence of *cag* PAI-positive strains. Within the U.S. and Western Europe, the prevalence of *cag* PAI-positive strains and *cag* PAI-negative strains is similar. In contrast, >90% of *H. pylori* strains isolated in many parts of East Asia (including Japan, Korea, and parts of China) are *cag* PAI-positive.[55,56] The predominance of *cag* PAI-positive strains in Japan, Korea, and parts of China correlates with a high rate of gastric cancer incidence in these geographic regions.[186]

The factors that determine the relative abundance of *cag* PAI-positive strains within populations are not known. In populations with high levels of *H. pylori* transmission, human stomachs can be colonized by multiple *H. pylori* strains, potentially leading to competition and eventual selection of strains that have the highest level of fitness. *cag* PAI-positive strains might have a selective advantage compared to *cag* PAI-negative strains in such settings. Conversely, *cag* PAI-negative strains might have a selective advantage in settings with low levels of *H. pylori* transmission and acquisition. Geographic variations in human genetic characteristics, diet, or other environmental factors might also influence the relative abundance of *cag* PAI-positive strains within geographic regions.

H. pylori strains isolated in different parts of the world are genetically distinct and have been classified into several different groups based on multi-locus sequencing typing (MLST) or genome-based analyses.[187] For example, *H. pylori* strains isolated from East Asian, European, and African human populations can be readily differentiated by genetic analysis. Among genes in the *cag* PAI, *cagA* sequences exhibit the highest level of geographic diversity.[9,188]

Some of the most striking geographic differences in CagA sequences are variations in the number and type of EPIYA phosphorylation site motifs.[189] CagA proteins produced by East Asian strains contain a type of EPIYA phosphorylation site motif (EPIYA-D) not detected in CagA proteins in other parts of the world.[78,190,191] Conversely, EPIYA-C motifs are commonly detected in CagA proteins produced by Western strains but not in CagA proteins produced by East Asian strains.[78,190,191] In vitro studies indicate that East Asian CagA proteins harboring the EPIYA-D motif exhibit activities that are different from those of CagA proteins lacking this motif. Specifically, CagA proteins containing a phosphorylated EPIYA-D motif bind SHP-2 with markedly higher affinity than CagA proteins containing the phosphorylated Western EPIYA-C motif, resulting in increased cellular morphologic alterations.[78,190,191] In contrast to the properties of CagA from East Asian

H. pylori strains, Cag proteins from Amerindian strains have a relatively low level of activity in vitro.[192]

Geographic differences in the prevalence of *cag* PAI-positive *H. pylori* strains likely contribute to geographic differences in gastric cancer incidence. Similarly, geographic differences in the characteristics of CagA EPIYA motifs probably influence gastric cancer incidence.[79] Additional geographic variations in the properties of *H. pylori* strains influencing T4SS activity may also be relevant. For example, one study reported that the *alpAB* genes contribute to T4SS-dependent IL-8 induction in East Asian strains but not Western strains.[155]

Summary and future directions

Colonization of the human stomach with *cag* PAI-positive strains is associated with an increased risk of gastric cancer and peptic ulcer disease, compared to colonization with *cag* PAI-negative strains. Similarly, experiments in multiple different animal models indicate that CagA and the Cag T4SS have important roles in the pathogenesis of *H. pylori*-induced gastric cancer and gastric ulceration. Experimental studies have revealed mechanisms by which proteins encoded by the *cag* PAI contribute to gastric disease.

Chronic inflammation promotes the development of cancer in multiple sites (for example, hepatocellular carcinoma associated with viral hepatitis, and colon cancer associated with inflammatory bowel disease). Therefore, chronic gastric mucosal inflammation stimulated by *cag* PAI-positive *H. pylori*, with associated DNA damage resulting from oxidative and nitrosative stress, is one of the important factors contributing to gastric cancer pathogenesis.[145,193]

CagA-induced alterations in cell signaling also contribute to gastric cancer pathogenesis. CagA-induced cellular alterations relevant for cancer pathogenesis include inhibition of apoptosis, stimulation of cell proliferation, degradation of the p53 tumor suppressor, and double-strand DNA breaks. Thus far, there has been relatively little progress in determining which types of cells in the gastric mucosa are targeted by CagA. Since differentiated superficial gastric epithelial cells are shed on a regular basis, CagA-induced alterations in these cells probably do not have a substantial impact on cancer pathogenesis unless CagA alters the behavior of these cells in a way that makes them resistant to shedding. Importantly, *H. pylori* can localize not only within the superficial gastric mucus layer, but also within gastric glands adjacent to gastric stem cells.[172] Targeting of gastric stem cells by CagA is presumed to have a key role in gastric cancer pathogenesis.[126,171,172]

Premalignant changes in the gastric environment, such as atrophic gastritis or intestinal metaplasia, render the stomach a relatively inhospitable environment for *H. pylori*. Therefore, by the time gastric cancer develops, *H. pylori* may no longer be detectable in gastric tissues. Since *H. pylori* and products of the *cag* PAI are not required for the maintenance of a cancer phenotype, it has been proposed that *H. pylori* and CagA can cause genetic or epigenetic alterations that persist in cells after CagA is no longer present, consistent with a "hit-and-run mechanism".[194]

Thus far, there has been relatively little progress in determining how the presence of the *cag* PAI benefits *H. pylori*. Within the human stomach, *cagA*-positive strains achieve a higher density than *cagA*-negative strains,[195] and CagA has been reported to promote *H. pylori* survival in human gastric organoids.[75] A fitness advantage conferred by the *cag* PAI would be especially relevant in settings where *cagA*-positive and *cagA*-negative strains co-colonize and compete within human stomachs, and might also enhance *H. pylori* transmission. A mechanism by which the *cag* PAI contributes to *H. pylori* fitness has not been thoroughly defined, but evidence from one study suggested that CagA contributes to bacterial iron acquisition.[196] In support of this hypothesis, *H. pylori* strains retain Cag T4SS activity in mice fed low-iron diets, but not in mice fed regular diets.[197]

We anticipate that continued studies of the *cag* PAI will lead to many important new discoveries that are relevant to human health and disease. For example, it will be important to further define mechanisms by which the Cag T4SS delivers multiple types of substrates into host cells and further define the cellular alterations that occur in response to these substrates. While multiple lines of evidence indicate that the *cag* PAI contributes to the pathogenesis of gastric disease, most individuals colonized with *cag* PAI-positive strains remain asymptomatic. Therefore, in future

studies, it will be important to define more completely the multiple additional bacterial, host, and environmental factors that determine gastric disease risk, so that individuals with the highest gastric cancer risk can be targeted for therapeutic intervention.

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