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The *Helicobacter pylori* Cag Type IV Secretion System

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Abstract

Colonization of the human stomach with *Helicobacter pylori* strains containing the *cag* pathogenicity island is a risk factor for development of gastric cancer. The *cag* pathogenicity island contains genes encoding a secreted effector protein (CagA) and components of a type IV secretion system (Cag T4SS). The molecular architecture of the *H. pylori* Cag T4SS is substantially more complex than that of prototype T4SSs in other bacterial species. In this review, we discuss recent discoveries pertaining to the structure and function of the Cag T4SS and its role in gastric cancer pathogenesis.

Keywords

type IV secretion system; bacterial protein secretion; bacterial nanomachines; macromolecular structures; cryo-electron microscopy; cryo-electron tomography; gastric cancer; gastric adenocarcinoma; peptic ulcer disease

Helicobacter pylori and gastric cancer

Helicobacter pylori, a Gram-negative spiral-shaped bacterium, persistently colonizes the stomach in about half of the global human population (1). While the majority never experience any adverse health consequences attributable to *H. pylori*, the presence of these bacteria in the stomach increases the risk for development of gastric adenocarcinoma and peptic ulcer disease (2). *H. pylori* infection is the strongest known risk factor for gastric cancer, and gastric cancer is the third leading cause of cancer-related death worldwide (3). Some *H. pylori* strains contain a 40-kb region of chromosomal DNA known as the *cag*

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pathogenicity island (*cag* PAI), whereas other strains do not (4-6). Individuals colonized with *H. pylori* strains containing the *cag* PAI have a higher risk of gastric adenocarcinoma and peptic ulcer disease compared to individuals colonized with *H. pylori* strains lacking the *cag* PAI (7-10). The *cag* PAI contains genes encoding a secreted effector protein (CagA) and multiple proteins required for delivery of CagA into host cells (11, 12). Several of the latter proteins exhibit low-level sequence relatedness to components of type IV secretion systems (T4SSs) in other bacterial species (Table 1)(12).

Recent studies have elucidated T4SS-dependent activities relevant to gastric cancer pathogenesis in cell culture systems and animal models. Other recent studies have used cryo-electron tomography (cryo-ET) to view the Cag T4SS *in situ* in intact *H. pylori* (13, 14) as well as single particle cryo-electron microscopy (cryo-EM) to determine the structure of T4SS complexes extracted from *H. pylori* (15, 16). These studies reveal that the molecular architecture of the *H. pylori* Cag T4SS is substantially more complex than that of prototype T4SSs in other bacterial species (Text Box 1). In this review, we discuss recent discoveries pertaining to the structure and function of the *H. pylori* Cag T4SS and its role in gastric cancer pathogenesis.

The *cag* pathogenicity island

The *cag* PAI is a 40-kb chromosomal region containing about 27 genes. These include *cagA* (encoding a secreted effector protein), 17 genes required for Cag T4SS activity and several genes with no known functions (4-6, 11, 12). *H. pylori* is the only Helicobacter species known to harbor the *cag* PAI. The %GC content of the *cag* PAI is lower than that of the rest of the *H. pylori* chromosome, which suggests that it was horizontally acquired. Geographically distinct populations of *H. pylori* throughout the world harbor the *cag* PAI (6), which suggests that it was horizontally acquired by ancestral *H. pylori* in the distant past, prior to human migrations out of Africa.

CagA effector protein

CagA was originally identified by detecting serum antibody responses to a high molecular mass (120-132 kDa) protein in humans colonized by *H. pylori* (17-20). Antibody responses to this protein are detected more commonly in individuals with gastric cancer or peptic ulcer disease than in asymptomatic *H. pylori*-positive individuals (7, 17, 18). The name "cytotoxin-associated gene A" (*cagA*) was originally assigned based on the observation that the 120-132 kDa antigenic protein and the gene encoding it are present more frequently in *H. pylori* strains with vacuolating toxin activity than in strains without detectable vacuolating toxin activity (19, 20). Subsequently it became clear that vacuolating toxin activity is mediated by the secreted VacA protein (21), which is encoded by a chromosomal locus distant from *cagA*, and *cagA* is not required for vacuolating toxin activity. Use of the term "*cag*" (cytotoxin-associated gene) when describing *cagA*, the *cag* PAI, and the Cag T4SS is based on the historical association of the vacuolating toxin phenotype with production of the 120-132 kDa antigenic protein.

Thus far, CagA is the only protein known to be secreted by the Cag T4SS. CagA is secreted and translocated into host cells by adherent *H. pylori* (Fig. 1), but it is not secreted into the extracellular space when the bacteria are cultured *in vitro* in the absence of host cells. The signals and regulatory mechanisms that trigger CagA secretion in response to *H. pylori* contact with host cells are not known. Upon entry into host cells, CagA undergoes phosphorylation by tyrosine kinases (Src and Abl) and interacts with at least 20 different host cell proteins, altering their activity (discussed in other reviews) (22-24).

The CagA proteins produced by different strains of *H. pylori* vary from about 120 to 145 kDa in mass. Most *H. pylori* contain a single copy of *cagA*, but some strains contain multiple tandem copies. CagA is characterized by a structured N-terminal region and an intrinsically disordered C-terminal region (25, 26). Both a C-terminal 20-amino-acid translocation signal and the N-terminal domain are required for efficient secretion of CagA (27, 28). The disordered C-terminal portion of the protein contains multiple sites (EPIYA motifs) where the protein undergoes phosphorylation by tyrosine kinases within host cells (29-31).

Transgenic mice expressing CagA (in the absence of *H. pylori* infection) develop gastric epithelial hyperplasia, gastric polyps, and adenocarcinoma of the stomach and small intestine, as well as myeloid leukemia and B-cell lymphoma (32). Similarly, CagA expression in Drosophila promotes increased cell proliferation, and transgenic zebrafish expressing CagA exhibit increased rates of intestinal epithelial cell proliferation (33). CagA induces intestinal small cell carcinoma and adenocarcinoma in zebrafish with a loss-of-function allele of p53 (33), and CagA expression in epithelial cells enhances the growth and invasion of tumors in a Drosophila model of metastasis (34). Therefore, multiple lines of evidence indicate that CagA has an important role in gastric carcinogenesis, leading to its designation as a “bacterial oncoprotein” (22, 23).

Proteins required for Cag T4SS activity

To define the genes required for Cag T4SS activity, *H. pylori* genes within the *cag* PAI have been individually mutated and the resulting mutant strains have been tested in T4SS functional assays. About 17 genes in the *cag* PAI are required for CagA translocation into gastric cells, and mutagenesis of several additional *cag* PAI genes results in a partial reduction in CagA translocation (Table 1)(11, 12). At least two *H. pylori* genes in chromosomal loci outside the *cag* PAI, encoding an outer membrane protein (HopQ)(35) and hydrogenase (36), influence Cag T4SS activity but are not considered components of the Cag T4SS.

Role of the Cag T4SS in *H. pylori*-induced alterations in host cells

Co-culture of gastric epithelial cells with *cag* PAI-positive *H. pylori* but not Cag T4SS mutant strains results in an assortment of cellular alterations, many of which are attributable to the actions of CagA. T4SS-dependent cellular alterations attributable to actions of CagA include changes in cell shape (resulting from cytoskeletal changes), disruption of cell-cell junctions, altered cell polarity and cell adhesion, increased cell motility and cell migration,

increased cell proliferation, β-catenin activation, and epithelial-mesenchymal transition (discussed in other reviews)(22-24).

H. pylori also causes T4SS-dependent changes in gastric epithelial cells that are not dependent on CagA. Among these, the most extensively studied phenomenon is stimulation of interleukin-8 (IL-8) production, which occurs through an NF-κB-dependent pathway. *H. pylori*-induced IL-8 production was initially attributed to the entry of peptidoglycan into host cells and recognition of peptidoglycan by Nod1 (Fig. 1)(37). More recently, this phenomenon has been attributed to the entry of lipopolysaccharide metabolites [heptose-1,7-bisphosphate (HBP) or ADP-glycero-β-D-manno-heptose (ADP-heptose), a derivative of HBP] into host cells and recognition of these molecules by tumor necrosis factor receptor-associated factor (TRAF)-interacting protein with forkhead-associated domain (TIFA), independent of Nod1 (38-41). Another T4SS-dependent alteration in host cells is activation of Toll-like receptor 9 (TLR9), a phenomenon attributed to the entry of *H. pylori* DNA into host cells (42). The *cag* PAI genes required for *H. pylori*-induced stimulation of IL-8 production in gastric epithelial cells or TLR9 activation are similar to those required for CagA translocation, but several genes required for CagA translocation [for example, CagF (a putative CagA chaperone) and Cagβ (an ATPase proposed to act as a coupling protein)] are not required for IL-8 production or TLR9 activation (11, 43-47).

In addition to the Cag T4SS, three additional *H. pylori* T4SSs have been described (known as the ComB T4SS, TFSS3 and TFSS4) (48). The ComB T4SS allows *H. pylori* to take up exogenous DNA through natural transformation. The functions of the TFSS3 and TFSS4 systems are not yet well understood. Thus far, there is no evidence that genes encoding components of the ComB T4SS, TFSS3 and TFSS4 are required for CagA translocation or other phenotypes associated with the Cag T4SS.

Role of the Cag T4SS in animal models of *H. pylori*-induced gastric disease

Mongolian gerbils infected with wild-type *cag* PAI-positive *H. pylori* strains develop severe gastric inflammation, often associated with gastric ulceration or gastric cancer (49-53). Mutant *H. pylori* strains with defects in Cag T4SS activity are able to colonize the gerbil stomach but cause less severe gastric inflammation and a much lower rate of gastric cancer or gastric ulceration than wild-type strains (49-53). Similar to T4SS mutant strains, CagA mutant strains cause less severe gastric inflammation and a markedly reduced rate of gastric cancer or gastric ulceration in a Mongolian gerbil model compared to isogenic wild-type strains (53-56). Therefore, the relative inactivity of Cag T4SS mutant strains is attributable, at least in part, to the failure of these strains to secrete CagA. Whether Cag T4SS-dependent delivery of non-protein substrates also contributes to gastric inflammation and carcinogenesis remains unclear.

Mice experimentally infected with wild-type *cag* PAI-positive *H. pylori* strains develop mild gastric inflammation but rarely develop gastric ulceration or gastric cancer. Cag T4SS and CagA mutant strains cause less severe gastric inflammation in mice compared to isogenic wild-type *cag* PAI-positive strains (57-59), but the differences are less striking than those observed in the Mongolian gerbil model. Wild-type *H. pylori* strains with an intact Cag

T4SS stimulate gastric stem cell expansion, resulting in increased cell proliferation and hyperplasia, whereas T4SS mutant bacteria do not (59, 60).

Molecular architecture of the Cag T4SS

Over the past decade, multiple models for the structural organization of the Cag T4SS have been proposed based on weak sequence relatedness of *cag* PAI-encoded proteins to components of T4SSs in other bacterial species (Table 1), localization of Cag proteins to specific subcellular fractions, experimentally identified protein-protein interactions among Cag proteins, and knowledge about T4SS architecture in other bacterial species. Generating models for the organization of the Cag T4SS has been challenging, however, because several Cag proteins do not exhibit sequence relatedness to components of T4SSs in other bacterial species (Table 1).

Two recent studies used cryo-electron tomography (cryo-ET) to reveal the structural organization of the Cag T4SS in intact bacteria (13, 14). When viewed by this approach, the Cag T4SS consists of a large mushroom-shaped structure that contacts the outer membrane, known as an outer membrane core complex (OMCC), as well as an inner membrane complex (IMC)(Fig. 2)(13, 14). The OMCC has 14-fold symmetry (13, 14). The IMC, consisting of three concentric rings surrounding a central channel, has six-fold symmetry (14). Mutant strains lacking individual ATPase components of the T4SS (CagE, Cagα and Cagβ) each exhibit detectable differences in IMC architecture compared to the wild-type strain (14).

The mushroom-shaped OMCC has been successfully extracted from *H. pylori*, thereby allowing analysis of its composition and structure (15, 16). The extracted complex is composed of 5 main components (CagY, CagX, CagT, Cag3 and CagM) (15). Three of these components (CagY, CagX and CagT) are homologous to OMCC components of T4SSs in other bacterial species, whereas Cag3 and CagM are present exclusively in *H. pylori* (Table 1). CagY, CagX and CagM are each required for OMCC assembly or stability (14, 15). OMCCs produced by Cag3 or CagT mutants have a narrower diameter than wild-type complexes (14, 15), which suggests that Cag3 and CagT are localized in the periphery of the complex.

Recently a 3.5 Å three-dimensional structure of isolated Cag T4SS particles was determined by single particle cryo-EM analysis (16). The particles were described as three sub-assemblies: an OMCC with 41 nm diameter, a periplasmic ring complex (PRC) with 18.5 nm diameter, and a central stalk (Fig. 3A). The OMCC can be further subdivided into an O-layer and an I-layer. A large cavity about 270 Å wide is present within the structure, extending from the bottom of the PRC to the top of the OMCC, where the cavity tapers to a 35 Å opening (Fig. 3B). The overall structure of Cag T4SS particles extracted from *H. pylori* appears similar to the mushroom-shaped periplasmic structures observed by cryo-ET (13-16). Interestingly, the cryo-ET studies visualized a “plug” structure within the large central cavity between the PRC and OMCC, as well as peripheral periplasmic densities, which were not visible in the high-resolution 3D reconstruction resulting from single particle cryo-EM analysis of isolated T4SS particles.

CagT, the C-terminal portion of CagY and the C-terminal portion of CagX are localized to the O-layer of the OMCC (Fig. 3). CagY localizes to the top of the OMCC, and a ring of CagY α-helices (two helices per protomer) is predicted to form a channel through the outer membrane. The structural features of putative transmembrane portions of CagY are markedly different from the typical β-barrel structures of most outer membrane proteins in Gram-negative bacteria. The exact protein composition of the I-layer, PRC, and central stalk has not yet been determined.

A recent model proposed that the Cag T4SS is built in a sequence beginning with assembly of a central cylinder (composed of CagY, CagX and maybe CagM), followed by incorporation of other OMCC components (CagT and Cag3), followed by assembly of the IMC (14). When viewed in cross-section, the OMCC has 14-fold symmetry, the PRC has 17-fold symmetry, and the IMC has 6-fold symmetry (14, 16). At present, very little is known about the protein-protein interactions that allow assembly of an apparatus with these multiple types of symmetry mismatch.

Structural comparisons of the *H. pylori* Cag T4SS and T4SSs in other bacterial species

Bacterial conjugation systems and the *A. tumefaciens* VirB/VirD4 T4SS are prototype T4SSs that have been studied extensively for several decades (Text Box 1)(61-64). In these systems, VirB7, VirB9 and VirB10 assemble into an OMCC, three ATPases and several other T4SS components localize to the inner membrane, and VirB2 and VirB5 localize to pilus structures (61-64). The OMCCs of conjugation systems encoded by pKM101 or R388 plasmids, the *A. tumefaciens* VirB/D4 system, and the *Xanthomonas citri* T4SS have been expressed in *E. coli*, extracted from the bacteria, and analyzed by single particle EM (electron microscopy) or crystallography (65-68). The OMCCs of these prototype T4SSs have outer and inner layers (O-layer and I-layer), each with 14-fold symmetry (65-68). VirB10 is predicted to span both the outer membrane and inner membrane. The outer membrane channel is formed by a portion of VirB10 (corresponding to a hydrophobic ring of two-helix bundles) (66). Lower resolution structures have been determined for a T4SS assembly encoded by the *E. coli* R388 conjugative plasmid, containing the OMCC connected by a stalk to an IMC (69). Within the IMC, 12 VirB4 ATPase subunits are organized as side-by-side hexameric barrels (69), and VirD4 localizes adjacent to VirB4 subunits (70).

The architecture of an F-plasmid-encoded T4SS (mediating bacterial conjugation) has been analyzed in intact bacteria using cryo-ET (71). Four distinct types of structures were visualized, presumably corresponding to different stages of T4SS biogenesis. The cryo-ET images provided an analysis of spatial relationships between the OMCC, IMC and pilus structures (71). In contrast to OMCCs of the prototype T4SSs described above, the OMCC of the F plasmid-encoded T4SS has 13-fold symmetry (71).

The *H. pylori* Cag T4SS exhibits structural similarities to the T4SSs described above, as well as notable differences (Fig. 4). Similarities include the overall shape, 14-fold symmetry of the OMCC, components with related sequences (Table 1), and conserved structural

features of the portions of CagY or VirB10 homologs that span the outer membrane. Conversely, the diameter of the *H. pylori* Cag T4SS OMCC is roughly twice that of OMCC diameters in prototype T4SSs (Fig. 4), and the Cag T4SS OMCC contains two proteins (Cag3 and CagM) that are not present in prototype T4SSs (15, 16). The presence of a distinct periplasmic ring complex (PRC) and symmetry mismatch between the OMCC and PRC are additional features that differentiate the Cag T4SS from prototype T4SSs (16).

The dimensions and number of components in the *H. pylori* Cag T4SS most closely resemble corresponding features of the *Legionella pneumophila* Dot/Icm T4SS (72, 73). The Legionella Dot/Icm potentially contains more than 20 components. Cryo-ET studies indicate that the OMCC of the Dot/Icm T4SS is about 42 nm in diameter (72, 73), which is similar to dimensions of the Cag T4SS OMCC and much larger than dimensions of OMCCs in prototype T4SSs. The Dot/Icm OMCC is predicted to contain at least 5 proteins (DotH, DotC, DotD, DotF and DotF). Cryo-ET analysis of the Dot/Icm T4SS suggests that it contains a subassembly similar to the PRC observed in the *H. pylori* Cag T4SS (72, 73), and there are also similarities in the structural organization of IMCs in the *H. pylori* Cag T4SS and the Legionella Dot/Icm T4SS (14, 72). In contrast to the 14-fold symmetry of the *H. pylori* Cag T4SS, the Legionella OMCC has 13-fold symmetry (72, 73). While the Cag T4SS and Dot/Icm T4SS exhibit multiple structural similarities, the sequences of individual proteins in these systems are highly divergent. One region of conserved sequence among OMCC components of the Legionella Dot/Icm T4SS, *H. pylori* Cag T4SS and prototype T4SSs is within the C-terminal portions of DotG/CagY/VirB10/TraF, which likely form a pore in the outer membrane (16).

“Pilus” structures associated with the *H. pylori* Cag T4SS

In response to contact with gastric epithelial cells, *H. pylori* produces extracellular filamentous structures (55, 74-82). An initial study reported that the structures were membrane-sheathed and produced more abundantly by bacteria co-cultured with cultured gastric epithelial cells than by bacteria cultured in the absence of epithelial cell contact (74). The structures were labeled “pili” based on the view that they were analogous to pilus components of conjugation systems and the *A. tumefaciens* VirB/VirD4 T4SS. Several studies reported that the pili are not produced by *H. pylori* strains lacking the *cag* PAI or harboring mutations in various genes encoding Cag T4SS components (74-76, 79, 80, 83). The dimensions of the structures described in various studies vary considerably (ranging from about 14 nm to 70 nm in width)(74, 76-79). Some studies reported that the structures are produced only when *H. pylori* is in contact with gastric epithelial cells, whereas other studies reported that the structures are also produced by bacteria in the absence of epithelial cell contact. A recent study reported that the structures form primarily when *H. pylori* contacts the basolateral surface of polarized epithelial cells, but not the apical surface (82). Several components of the Cag T4SS, including CagY, CagT and CagL, have been localized to the structures using immunogold EM (74, 76), and CagA has been localized to the tip of the structures (76, 78). The structures have never been isolated, so their biochemical composition has not been defined.

Cryo-ET methods were used recently to analyze *H. pylori* in contact with gastric epithelial cells, and membranous tube-like structures (about 37 nm in diameter) protruding from the outer membrane were visualized (13). The structures were produced by a wild-type strain but not a *cag* PAI mutant strain (13), and were observed only when the bacteria were co-cultured with gastric epithelial cells but not in bacteria grown in pure culture. Interestingly, the membranous tubes visualized by cryo-ET were not detected in direct association with T4SS OMCCs.

While many studies have concluded that the extracellular structures are pilus components of the Cag T4SS, several gaps in knowledge and apparent inconsistencies need to be addressed before this view can be fully accepted. First, a major pilin subunit has not been conclusively identified. Second, the lack of close proximity between the filaments and T4SS OMCCs in cryo-ET images (13) raises questions about whether there is any relationship between these structures. Finally, it is notable that *H. pylori* strains containing deletion or insertion mutations in several genes required for Cag T4SS activity (*cagC, cagH* and *cagY*) produce extracellular pilus structures, similar to a wild-type strain (79, 80, 83). CagY (a VirB10 homolog) would likely be required for export of pilus components (80, 83). In future studies, it will be important to define the composition of the pili, filaments or tubes, clarify if one or more than one type of structure is produced, and more rigorously determine the genetic requirements for production of these structures.

H. pylori Cag T4SS interactions with the surface of host cells

Relatively little is known about interactions between the Cag T4SS and host cells. CagL, a T4SS component localized to pilus structures and the bacterial surface, is recognized by TLR5 on host cells, resulting in flagellin-independent activation of TLR5 and NF-κB activation (84). In addition, multiple components of the T4SS (CagL, CagY and CagI) as well as CagA interact with integrins (α5β1 as well as other types), and it has been proposed that integrins might be host cell receptors for the T4SS (26, 76, 78, 85, 86). Interactions of the T4SS with integrins could potentially be mediated by either Cag proteins localized on the surface of *H. pylori* or by proteins localized to pilus structures. A ring of CagY α-helices localized at the apex of the OMCC is predicted to form a pore through the *H. pylori* outer membrane (16) and would likely be accessible for interactions with integrins or other host cell receptors. Integrins are typically localized on the basolateral surface of gastric epithelial cells, so it has been suggested that secreted *H. pylori* proteases may allow the bacteria to breach cell-cell junctions and gain access to integrins on the basolateral surface of cells (82). While multiple studies have detected interactions between the components of the Cag T4SS and integrins, one study reported that integrins were not required for CagA translocation, and instead, CEACAM proteins (receptors for the *H. pylori* HopQ protein) were required (87).

Whether components of the T4SS insert into the plasma membrane of host cells to form a channel for translocation of CagA and other *H. pylori* constituents is unknown. If a component of the T4SS inserts into the plasma membrane (analogous to the translocon of T3SSs), the protein responsible for such an action has not yet been identified. One model proposed that adherence of *H. pylori* to gastric epithelial cells stimulates externalization of

phosphatidylserine to the outer leaflet of the plasma membrane, and proposed that binding of CagA to phosphatidylserine then triggers internalization of CagA through an energy-dependent process distinct from receptor-mediated endocytosis (88). In this model, CagA translocation through the bacterial envelope is proposed to be mediated by the Cag T4SS, and CagA internalization into host cells is proposed to occur without a requirement for T4SS-dependent permeabilization of the plasma membrane (88, 89).

Concluding remarks and future perspectives

Type IV secretion systems are complex molecular machines found in many bacterial species. Our current understanding of these machines has come mainly from studies of conjugation systems and the *A. tumefaciens* VirB/VirD4 T4SS. Although multiple features of these prototype T4SSs are broadly conserved, there is extensive variation in the structure and function of T4SSs from different bacterial species. The *H. pylori* Cag T4SS is of particular interest because of its role in the pathogenesis of gastric cancer. Over the past 25 years since the potential existence of a secretion system encoded by the *cag* PAI was first recognized (4, 5, 90), there has been a steady increase in our understanding of the Cag T4SS. Cryo-ET analyses have provided low resolution models of the Cag T4SS OMCC and IMC in the context of intact *H. pylori* cells (13, 14), and single particle cryo-EM analysis has provided a high resolution model of the OMCC and PRC, as well as a molecular model for the positions of CagY, CagX and CagT in the OMCC (16) (Fig. 3). These structural studies combined with biochemical and functional analyses provide a strong foundation for further investigation (see “Outstanding Questions”).

Assembly of the Cag T4SS is energetically costly and would be unfavorable unless the secretion system conferred substantial benefits to the bacteria. The Cag T4SS is not required for *H. pylori* colonization of the human stomach but it likely confers competitive advantages. *In vitro* studies suggest that CagA enhances the capacity of *H. pylori* to extract iron and other nutrients directly from polarized gastric epithelial cells (91, 92) or access nutrients by disrupting intercellular junctions (93), thereby promoting bacterial growth. Gastric inflammation stimulated by T4SS-dependent processes may also enhance availability of nutrients utilized by *H. pylori*. CagA and the Cag T4SS inhibit expression of β-defensin 1 and β-defensin 3, thereby allowing *H. pylori* to evade innate host defenses (94, 95). Enhanced ability to obtain nutrients and resist host defenses are two possible factors contributing to an increased density of gastric colonization observed with CagA-positive *H. pylori* strains compared to CagA-negative strains (96). Actions of the Cag T4SS might allow *H. pylori* to colonize expanded gastric niches (for example, the gastric corpus)(50). In addition, T4SS-dependent actions can potentially lead to alterations in the gastric microbiome or intestinal microbiome (97, 98). Collectively, actions of the Cag T4SS are likely to enhance *H. pylori* growth in the stomach and enhance transmission of *H. pylori* to new hosts. In future studies, it will be important to further delineate mechanisms by which the Cag T4SS confers benefits to *H. pylori*. In addition, it will be important to develop a clearer understanding of not only the mechanisms by which Cag T4SS-positive *H. pylori* strains contribute to the pathogenesis of gastric cancer and peptic ulcer disease, but also mechanisms by which these strains potentially confer health benefits to human hosts.

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Glossary

Helicobacter pylori
Gram negative bacteria that colonize the human stomach

***cag* pathogenicity island (PAI)**
a region of chromosomal DNA, present in some *H. pylori* strains but not others, which contains genes encoding CagA and components of a T4SS

T4SS (type IV secretion system)
a diverse family of bacterial secretion systems with multiple possible functions (for example, delivery of effector proteins into target cells or bacterial conjugation)

Cag T4SS
a T4SS composed of proteins encoded by genes in the *H. pylori cag* PAI

CagA
the only known effector protein transported by the Cag T4SS, designated as a bacterial oncoprotein

Interleukin-8 (IL-8)
a proinflammatory cytokine

Dot/Icm T4SS
a type of T4SS present in *Legionella pneumophila* (causative agent of Legionnaire's disease)

VirB/VirD4 T4SS
a prototype form of T4SS present in *Agrobacterium tumefaciens* (which delivers T-DNA into plant cells, resulting in crown gall disease)

Conjugation systems
class of T4SSs (composed of Tra components) that mediate DNA exchange between bacteria

Outer membrane core complex (OMCC)
a T4SS subassembly localized within the periplasm, in contact with the outer membrane

Periplasmic ring complex (PRC)
a T4SS subassembly localized within the periplasm

Inner membrane complex (IMC)
a T4SS subassembly containing ATPases, localized in association with the inner membrane and extending into the cytoplasm

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Outstanding questions

1. How does the T4SS apparatus assemble? Where are individual proteins localized with the T4SS and what are their functions? If the Cag T4SS is a dynamic structure, how many structural states can be identified?
2. T4SSs found in *H. pylori* and Legionella are larger in size and more complex than prototype T4SSs. In what ways do increased size and complexity contribute to the function of these systems?
3. How are CagA and non-protein *H. pylori* constituents (LPS metabolites, peptidoglycan and DNA) recruited to the T4SS?
4. Does CagA transit through the central chamber within the T4SS? Is it translocated in a folded or unfolded state?
5. What are the stimuli associated with bacteria-host cell contact that trigger CagA secretion, and what are the corresponding regulatory mechanisms?
6. By what mechanisms do CagA and non-bacterial constituents transit the plasma membrane of host cells?
7. What is the role of extracellular structures (pili or membranous tubes) in T4SS-mediated processes?
8. What benefits are conferred to *H. pylori* by the Cag T4SS?
9. The Cag T4SS contributes to gastric cancer and peptic ulcer disease in patients colonized with *H. pylori*. Do *H. pylori* strains producing the Cag T4SS also confer health benefits to humans?

Highlights

1. The Cag T4SS has a key role in the pathogenesis of *H. pylori*-associated gastric cancer.
2. Recent advances in molecular imaging have facilitated investigation of *H. pylori* Cag T4SS structure.
3. The *H. pylori* Cag T4SS contains multiple components unrelated to components of T4SSs in other bacterial species, and the molecular architecture of the *H. pylori* Cag T4SS is substantially more complex than that of prototype T4SSs.
4. T4SSs are a heterogenous group of secretion systems with diverse functions. Despite a low level of sequence relatedness among corresponding components of T4SSs from different bacterial species, there are shared structural features among all T4SSs analyzed thus far.

Text Box 1.

"Overview of bacterial type IV secretion systems"

Bacteria utilize more than 10 different types of secretion systems to translocate proteins across the cell envelope (99). Type IV secretion systems (T4SSs) are complex and functionally diverse molecular machines found in both Gram-negative and Gram-positive bacteria as well as Archaea (61-64). In Gram-negative bacteria, T4SSs span both the inner and outer membrane. T4SSs can carry out a wide range of functions, including delivery of effector proteins into eukaryotic or bacterial target cells, delivery of DNA into target cells, and import (or export) of substrates from (or to) the extracellular environment (61-64, 100-102). Two of the most extensively studied examples of T4SSs are bacterial conjugation systems, which mediate DNA exchange between bacteria, and the *Agrobacterium tumefaciens* VirB/VirD4 T4SS, which delivers bacterial T-DNA into plant cells, resulting in tumor formation (crown gall disease). T4SS-mediated delivery of effector proteins into host cells is required for the pathogenicity of many bacterial species, including *H. pylori, Legionella pneumophila, Bordetella pertussis, Coxiella, Brucella*, and *Bartonella*. In most (but not all) cases, T4SS-mediated delivery of effector proteins into recipient cells is contact-dependent. T4SSs can be phylogenetically grouped into two main families: type IVA (exemplified by conjugation systems, the *A. tumefaciens* VirB/VirD4 T4SS, and the *H. pylori* Cag T4SS) and type IVB (exemplified by the Legionella Dot/Icm T4SS)(102). "Minimized" T4SSs, exemplified by conjugations systems and the *A. tumefaciens* VirB/VirD4 T4SS, have about 12 components (VirB1-11 and VirD4). In contrast, the *H. pylori* Cag T4SS and the *Legionella* Dot/Icm T4SS are more complex and have a substantially larger number of components.

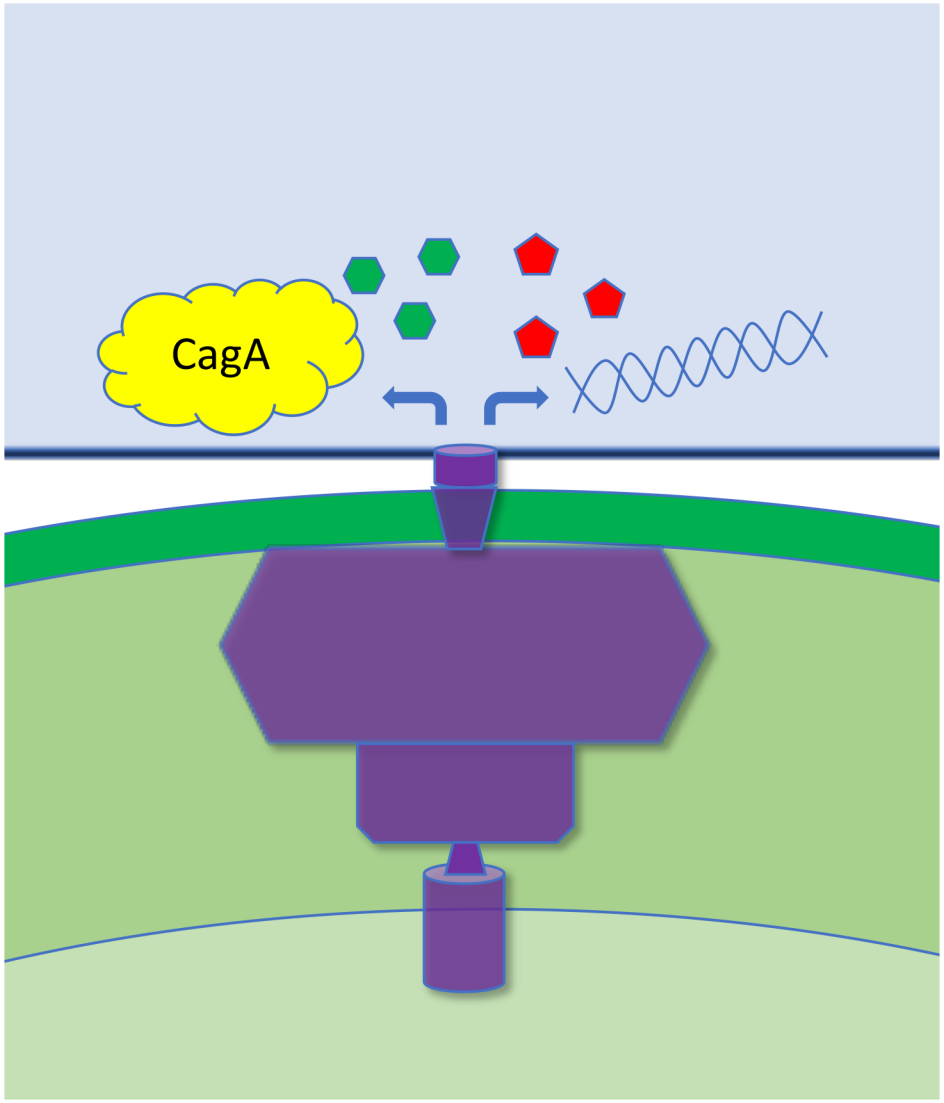

Fig. 1.

Schematic depicting an *H. pylori* bacterium adhering to a host cell. The Cag T4SS (purple) facilities delivery of the CagA effector protein into host cells, as well as intracellular entry of *H. pylori* LPS metabolites, peptidoglycan and DNA.

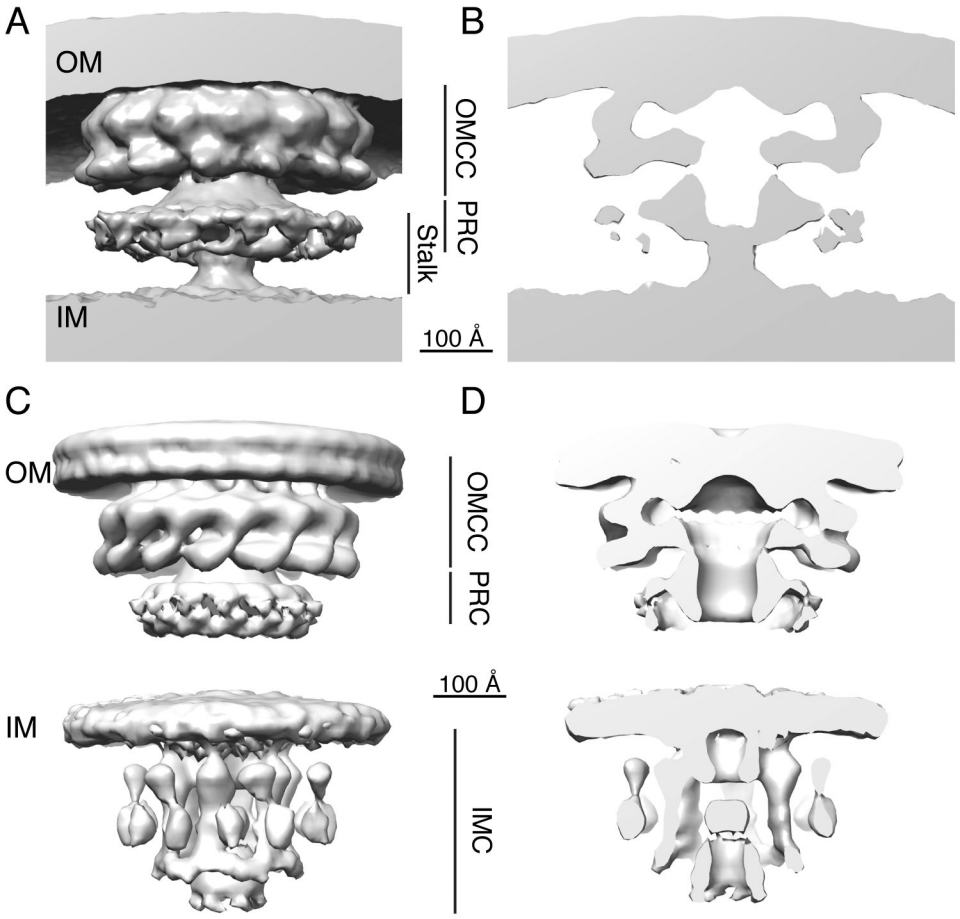

Fig. 2.

Cryo-ET analyses of the *H. pylori* Cag T4SS. Spatial relationships between the T4SS, the bacterial outer membrane (OM) and inner membrane (IM) are shown. (A,B) Cryo-ET analysis of *H. pylori* attached to AGS gastric cells, showing the 3D structure and a central axial slice view of the 3D structure (EMD-7474)(13). Scale bar, 100 Å. (C,D). Cryo-ET analysis of *H. pylori* grown in pure culture (without AGS gastric cells) showing the 3D structure and a central axial slice view of the 3D structure (EMD- 0634 and 0635)(14). Scale bar, 100 Å. The outer membrane core complex (OMCC), periplasmic ring complex (PRC), stalk, and inner membrane complex (IMC) are labeled.

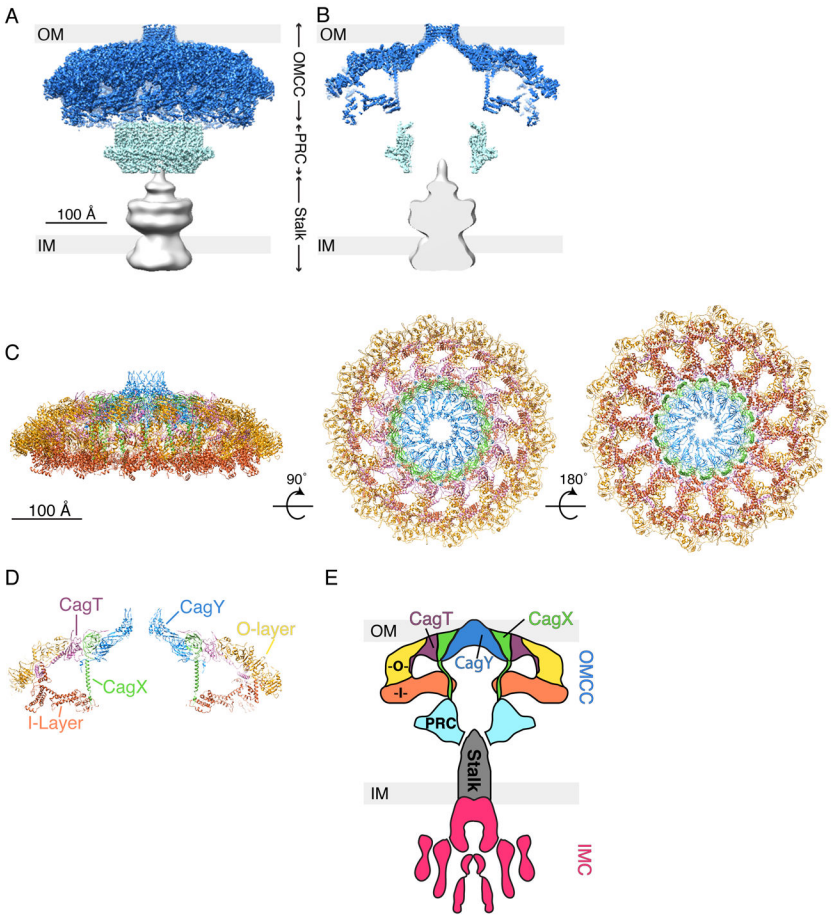

Fig. 3.

3D structure of Cag T4SS particles extracted from *H. pylori* and determined by single particle cryo-EM. (A) 3D reconstruction of the Cag T4SS with the predicted position of the bacterial outer membrane (OM) and inner membrane (IM) shown (EMD-20023, 20020, and 20021) (16). Outer membrane core complex (OMCC), blue; periplasmic ring complex (PRC), cyan; and stalk, grey. (B) Central axial slice view of the 3D structure in panel A. (C) Secondary structure model of the Cag T4SS OMCC showing the positions of CagT (purple, PDB-6OEE), the C-terminal portion of CagX (green, PDB-6OEG), and the C-terminal portion of CagY (blue, PDB-6ODI), as well as unassigned outer-layer (O-layer, yellow, PDB-6OEF) and inner-layer (I-layer, orange, PDB-6OEH) densities (16). The structure is rotated 90° and then 180° around the X-axis. (D) Central slice view of the structure shown in panel C (left image). (E) Schematic summarizing our current understanding of Cag T4SS architecture, based on cryo-ET and single particle EM studies (13, 14, 16). The Cag T4SS is composed of an OMCC, periplasmic ring complex (PRC, cyan), stalk (grey), and inner membrane complex (IMC, magenta). The positions of CagT (purple) and portions of CagX (green) and CagY (blue) have been mapped into the OMCC. There are outer and inner layer densities (O, yellow and I, orange) that have yet to be assigned a specific T4SS component. The identities and position of T4SS components in the PRC and stalk also have not yet been structurally determined.

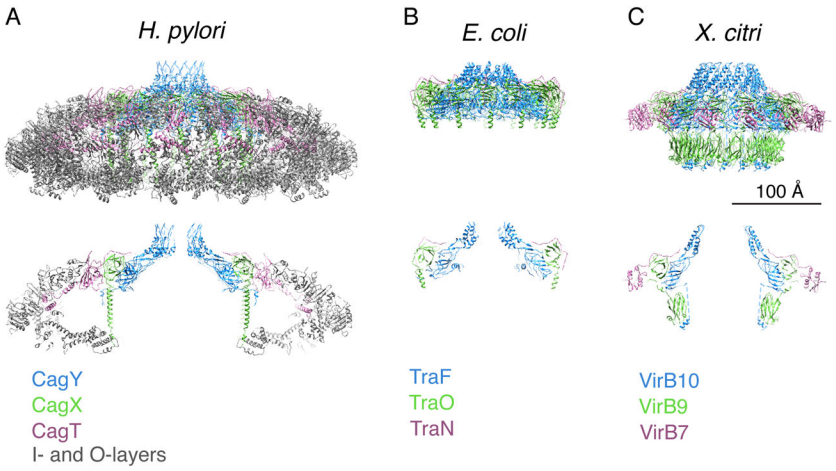

Fig. 4.

Structural comparison of the *H. pylori* Cag T4SS OMCC with minimized T4SSs from other bacterial species. (A-C) Upper panel, secondary structural models of the complexes. Lower panel, Central axial slice views of the secondary structural models. Scale bar, 100 Å. (A) OMCC from the *H. pylori* Cag T4SS (16). CagY (PDB-6ODI, blue), CagX (PDB-6OEG, green), CagT (PDB-6OEE, purple), and outer- and inner-layer proteins (PDB-6OEF and -6OEH, grey). (B) Conjugation system (encoded by pKM101) (66). TraF (PDB-3JQO, blue), TraO (PDB-3JQO, green), and TraN (PDB-3JQO, purple). (C) *Xanthomonas citri* T4SS (67). VirB10 (PDB-6GYB, blue), VirB9 (PDB-6GYB, green), and VirB7 (PDB-6GYB, purple).

Table 1.

cag PAI-encoded proteins required for Cag T4SS activity[a]

Gene number[b]	Protein name	Localization[c]	Predicted localization[d]	Structure[e]	Homologs	Putative function
Proteins with defined localization in T4SS apparatus						
hp0528	CagX	OMCC		6OEG 5H3V	VirB9	
hp0527	CagY	OMCC		6ODI	VirB10	
hp0532	CagT	OMCC		6OEE	VirB7	
hp0537	CagM	OMCC				
hp0522	Cag3	OMCC				
hp0525	Cagα	IMC		2PT7 1G6O	VirB11	ATPase
hp0524	Cagβ	IMC			VirD4	ATPase
hp0544	CagE	IMC			VirB3/VirB4	ATPase
Proteins for which localization in T4SS apparatus is not yet defined						
hp0546	CagC		IM, OM, S		VirB2	
hp0545	CagD[f]		IM, PP, S	3CWX		
hp0543	CagF		IM, C			CagA chaperone
hp0542	CagG[f]		PP			
hp0541	CagH		IM			
hp0540	CagI		PP			
hp0539	CagL		PP, S	4CII 4YVM 3ZCI		
hp0538	CagN[f]		PP, IM			
hp0531	CagU		IM			
hp0530	CagV		IM	6IQT	VirB8	
hp0529	CagW		IM			
hp0526	CagZ[f]		IM	1S2X		
hp0523	Cag4		PP		VirB1	PG hydrolase
Secreted effector protein						
hp0547	CagA		C,S	4DVY 4GOH		Effector protein

[a]The proteins listed are required for CagA translocation into host cells (11, 12). Several proteins (for example, CagF and Cagβ) are required for CagA translocation into host cells but are not required for other T4SS-dependent phenotypes (such as stimulation of IL-8 production) (11, 43-47).

[b]Gene number in *H. pylori* strain 26695

[c]Location in T4SS apparatus based on biochemical analysis of extracted subassemblies, cryo-ET analysis or cryo-EM analysis. OMCC: outer membrane core complex. IMC: inner membrane complex.

[d]Predicted localization based on features of protein sequences or fractionation experiments. OM, outer membrane; IM, inner membrane; C, cytoplasm; S, surface-exposed or supernatant; PP, periplasm.

[e] Protein database (PDB) IDs for structures are listed.

[f] Conflicting results about requirement for T4SS activity, or mutants reported to exhibit a partial reduction in T4SS activity.