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Activity and Functional Importance of *Helicobacter pylori* Virulence Factors

Dionyssios Sgouras, Nicole Tegtmeyer, and Silja Wessler

Abstract

Helicobacter pylori is a very successful Gram-negative pathogen colonizing the stomach of humans worldwide. Infections with this bacterium can generate pathologies ranging from chronic gastritis and peptic ulceration to gastric cancer. The best characterized *H. pylori* virulence factors that cause direct cell damage include an effector protein encoded by the cytotoxin-associated gene A (CagA), a type IV secretion system (T4SS) encoded in the *cag*-pathogenicity island (*cag* PAI), vacuolating cytotoxin A (VacA), γ-glutamyl transpeptidase (GGT), high temperature requirement A (HtrA, a serine protease) and cholesterol glycosyl-transferase (CGT). Since these *H. pylori* factors are either surface-exposed, secreted or translocated, they can directly interact with host cell molecules and are able to hijack cellular functions. Studies on these bacterial factors have progressed substantially in recent years. Here, we review the current status in the characterization of signaling cascades by these factors *in vivo* and *in vitro*, which comprise the disruption of cell-to-cell junctions, induction of membrane rearrangements, cytoskeletal dynamics, proliferative, pro-inflammatory, as well as, pro-apoptotic and anti-apoptotic responses or immune evasion. The impact of these signal transduction modules in the pathogenesis of *H. pylori* infections is discussed.

Keywords

E-cadherin · Protease · CagA · HtrA serine protease · VacA · UreA · Adherens junction · Tight junction · Epithelial barrier · Type IV secretion T4SS

D. Sgouras (✉)
Laboratory of Medical Microbiology, Hellenic Pasteur Institute, Athens, Greece
e-mail: sgouras@pasteur.gr; dionsgouras@gmail.com

N. Tegtmeyer
Division of Microbiology, Department of Biology, Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany
e-mail: nicole.tegtmeyer@fau.de

S. Wessler
Division of Microbiology, Department of Biosciences, Paris-Lodron University of Salzburg, Salzburg, Austria
e-mail: silja.wessler@sbg.ac.at

1 Introduction

The human stomach represents a highly dynamic and hostile environment for bacteria, in which the gastric pathogen *H. pylori* encounters numerous stresses, including nutrient limitations, pH fluctuations or oxidative attack (Kusters et al. 2006). Gastric colonization by *H. pylori* commonly occurs in early childhood and can persist for the entire lifetime, unless it is eradicated by antimicrobial treatment. The bacterium is a major risk factor for the development of various gastric

diseases and severe disorders, such as peptic ulcer disease, that can develop in about 10–15%, or gastric malignancies in 1–2% of infected individuals; occurrence of these pathologies depends on complex host-pathogen interactions and correlates to the geography of individuals (Polk and Peek 2010; Yamaoka and Graham 2014). The presence of *H. pylori* in the stomach mucosa is commonly accompanied by strong inflammatory responses, however, several immune evasion strategies by the pathogen have been described (Mejias-Luque and Gerhard 2017) presenting a prime example of a chronic bacterial infection (Ramarao et al. 2000; Pachathundikandi et al. 2016). About half of the human world population is colonized by the pathogen, associated with chronic or asymptomatic gastritis in every infected person. The pathogen has evolved multiple mechanisms to colonize and persist within the human stomach despite the harsh acidic conditions confronted in this milieu (Robinson et al. 2017). *H. pylori* is highly adapted to the stomach and grows at pH ranges between 6 and 8. Physiological, biochemical and genetic studies of *H. pylori* have identified unique properties of its metabolism, some of which are crucial for the adaptation to the gastric environment (Kusters et al. 2006). Well-known pathogenicity-associated properties of *H. pylori* comprise flagella-mediated motility, urease-driven chemotaxis and neutralization of gastric pH, counteraction of antimicrobial nitric oxide production by arginase RocF and binding of the bacteria to gastric epithelial cells using several outer-membrane proteins; the latter adhesins include BabA/B, SabA, AlpA/B, OipA, HopZ, HopQ, and others (Gobert et al. 2001; Dubois and Borén 2007; Backert et al. 2011; Roure et al. 2012; Posselt et al. 2013; Huang et al. 2015; Naumann et al. 2017).

Genetic studies have shown that *Homo sapiens* has carried *H. pylori* for more than 100,000 years and DNA sequence characteristics of the bacteria were utilized as signatures to outline multifaceted demographic events in the history of mankind, including major human migration routes (Moodley and Linz 2009). Because of this long time of co-existence with humans, it was proposed that hosting of *H. pylori* may have been advantageous for its carrier (Atherton and Blaser 2009). In our modern civilization, however, the bacterium produces a strong burden of morbidity and mortality caused by malignancies such as gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Polk and Peek 2010; Figueiredo et al. 2017). Gastric cancer represents one of the highest incident malignancies on the planet, causing over 700,000 deaths annually (Ferlay et al. 2015). The clinical consequences of *H. pylori* infection are controlled by a very complicated setup of host-pathogen interactions. The infection and development of gastric diseases is dependent on multiple parameters, including environmental factors, genetic predisposition of the host and bacterial virulence determinants. For example, the stomach microbiota, various dietary aspects, as well as important micronutrients can influence and change the equilibrium between *H. pylori*'s endeavor as a pathogen or a commensal (Amieva and El-Omar 2008; Polk and Peek 2010; Yamaoka and Graham 2014). Moreover, specific single nucleotide polymorphisms (SNPs) have been discovered in pro-inflammatory and other immune-regulatory control genes of the human genome, including tumor necrosis factor, interleukin-1β, interleukin-8, Nod-like and toll-like receptors, which can account for an increased risk of developing gastric diseases induced by *H. pylori* (Amieva and El-Omar 2008). Commonly, *H. pylori* isolates are genetically extremely variable, and this diversity also includes the presence of virulence genes, revealing different degrees of pathogenicity that affects the severity of *H. pylori* infections. Molecular mechanisms evolved in *H. pylori* to challenge host defense instruments and causing disease are under vigorous examination, by numerous research labs worldwide. Here, we review the function and activity of the major *H. pylori* virulence factors *cag* PAI carrying T4SS and CagA, VacA, HtrA, GGT and CGT.

2 Assembly and Function of the *cag* PAI-Encoded T4SS

The *cag* PAI is a genetic locus of ~40 kilobase pairs in the *H. pylori* chromosome carrying up to 32 genes that was acquired from a yet unknown ancestor by horizontal DNA transfer (Covacci and Rappuoli 2000). The *cag* PAI is present in highly virulent (type-I) *H. pylori* isolates but typically absent in less virulent (type-II) strains. Functional studies have shown that the *cag* PAI encodes a T4SS, representing a syringe-like nanostructure, spanning the inner and outer membranes of the Gram-negative bacterium. T4SS assembly involves orthologs of all 12 VirB/VirD4 subunits that were first described for the prototype *Agrobacterium tumefaciens* apparatus, and about a dozen additional Cag PAI proteins, making this system clearly unique among other T4SSs as discussed elsewhere (Backert et al. 2015; Grohmann et al. 2018). Electron microscopy has been applied to visualize the T4SS core structure which is sized approximately 41 nm in diameter, and comprises a complex of the Cag3, CagT, CagM, CagX and CagY proteins (Frick-Cheng et al. 2016). This core structure is connected with an extracellular pilus appendage in the outer membrane, which establishes host cell contact (Kwok et al. 2007; Shaffer et al. 2011). The CagL, CagI, CagY and CagA proteins have been identified as pilus-linked factors and permit binding to the host receptor integrin $\alpha_5\beta_1$, which is necessary for T4SS functionality (Kwok et al. 2007; Jimenez-Soto et al. 2009; Barden et al. 2013). The integrin $\alpha_v\beta_5$ member was also found to be exploited by *H. pylori* to induce gastrin production in a T4SS-dependent fashion (Wiedemann et al. 2012). Various translocated effector molecules and signaling effects have been reported (Fig. 1). The T4SS injects effector protein CagA (Segal et al. 1999; Stein et al. 2000; Odenbreit et al. 2000; Asahi et al. 2000; Backert et al. 2000), peptidoglycan (Viala et al. 2004), chromosomal DNA (Varga et al. 2016) and heptose-1,7-bisphosphate (HBP) into epithelial target cells, which respectively can stimulate receptor Nod1, toll-like receptor-9, TRAF-interacting protein with FHA domain (TIFA), kinase AKAP and pro-inflammatory transcription factor NF-κB in infected epithelial cells (Viala et al. 2004; Varga et al. 2016; Gall et al. 2017; Stein et al. 2017; Zimmermann et al. 2017).

Interestingly, *H. pylori* also targets the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors by means of adhesin HopQ for bacterial adhesion and delivery of CagA (Fig. 1) (Javaheri et al. 2016; Koniger et al. 2016). It appears that HopQ exploits the CEACAM dimer interface for binding and this interaction is required for proper T4SS function of yet unknown nature, which is required for effective stomach colonization and subsequent gastric pathogenesis (Bonsor et al. 2018; Moonens and Remaut 2017). In addition, the T4SS itself can also interact with and activate specific other host cell receptors in a CagA-independent manner, including epidermal growth factor receptor members EGFR and Her2/Neu, leading to increase cellular proliferation, anti-apoptosis and bacterial survival (Keates et al. 2001; Saha et al. 2010; Tegtmeyer et al. 2010; Sierra et al. 2018). Furthermore, the T4SS stimulates the receptor tyrosine kinase c-Met, which induces epithelial cell migration and invasion by engaging phospholipase PLCγ and mitogen-activated kinases (Fig. 1) (Churin et al. 2003; Oliveira et al. 2006).

Early studies have shown that *H. pylori* can actively inhibit its phagocytosis through professional phagocytes (Ramarao et al. 2000). These antiphagocytosis characteristics possibly play an important role in immune escape of *H. pylori* and depend on a functional *cag* PAI, since isogenic T4SS mutants abolished this feature, but does not require CagA (Ramarao et al. 2000). In addition, the pathogen was described to change the phosphorylation state of histone H3 through a CagA-independent but T4SS-dependent mechanism involving the mitotic vaccinia-related kinase 1 and Aurora B (Fehri et al. 2009). Remarkably, in epithelial cells T4SS-positive bacteria can also stimulate the NF-κB-mediated induction of AID (a DNA-editing enzyme) that leads to the

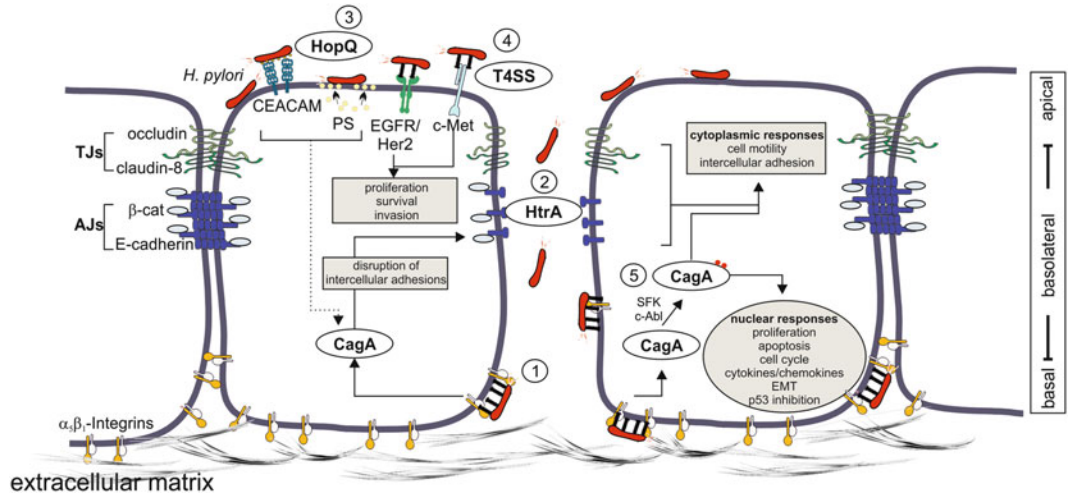

Fig. 1 T4SS-dependent effects in *H. pylori*-infected cells. Polarization of the gastric epithelium involves functional intercellular adhesion complexes, such as tight junctions (TJs) and adherens junctions (AJs). Disruption of the epithelium is facilitated by T4SS-positive *H. pylori* strains. The T4SS contact α5β1-integrins to inject the virulence factor CagA into the cytosol of infected cells (*1*), which can compete with β-catenin (β-cat) binding to the intracellular domain of the AJ cell adhesion molecule E-cadherin and contribute to the disruption of AJs. CagA translocation is enhanced by the *H. pylori*-secreted serine protease HtrA. HtrA cleaves off the extracellular domains of E-cadherin, occludin, and claudin-8 (*2*), which opens intercellular TJs and AJs. HtrA-mediated cleavage of adhesion molecules further allows binding of the T4SS to the α5β1-integrins at the basolateral domain of polarized cells. HopQ interaction with apically expressed CEACAMs is involved in efficient CagA injection (*3*). Furthermore, the T4SS can directly target receptors on the cell surface, including EGFR, Her2/Neu or c-Met, which is implicated in proliferation, cell survival and invasive growth (*4*). Cytoplasmic CagA is finally tyrosine-phosphorylated by kinases of the Src (SFK) and Abl family (*5*). Both phosphorylated and non-phosphorylated CagA induce changes in nuclear responses (e.g., proliferation, apoptosis, cell cycle arrest, synthesis of cytokines and chemokines, induction of EMT or p53 inhibition). Lastly, CagA may interfere with signaling pathways leading to cell motility, which might be facilitated by the disintegrated AJs and TJs

accumulation of mutations in p53, a well-known tumor suppressor protein (Matsumoto et al. 2007). Therefore, the activation of AID could represent a mechanism in which mutations in crucial genes could accumulate during infection and trigger gastric malignancy. Finally, the T4SS of *H. pylori* infection engages glycoprotein receptor gp130 (Lee et al. 2010), and the downstream activation of JAK2–STAT3 (Janus kinase–signal transducer and activator of transcription) signaling is linked to *H. pylori*-induced inflammation, which promotes carcinogenesis. Taken together, the T4SS located on the *cag* PAI exhibits remarkable features in its interactions with the host and is involved in causing gastric pathology. These data also demonstrate that *H. pylori* disrupts crucial cellular processes by one or more yet unidentified T4SS factors, which need to be identified in future studies.

3 CagA, a Multifunctional Master Key

CagA is an extraordinary protein of approximately 120–140 kDa, not sharing any sequence homology with other proteins known to date. It represents the most researched *H. pylori* virulence factor with over 3200 citations in PubMed (Backert and Blaser 2016). It was originally identified independently by two groups as an immunodominant protein of about 128 kDa in seropositive *H. pylori* carriers (Tummuru et al. 1993; Covacci et al. 1993). Subsequently, CagA-seropositivity in symptomatic patients was found to be associated with increased risk of gastric cancer (Blaser et al. 1995; Parsonnet et al. 1997). Its biological importance was further acknowledged when a number of research groups

reported that CagA can be translocated into gastric epithelial cells, passing the membrane by means of T4SS (Covacci and Rappuoli 2000; Backert and Tegtmeyer 2017). Further work convincingly demonstrated that for the successful translocation of the CagA protein, interaction of a number of T4SS constituents with host receptor integrin α5β1 was necessary (Kwok et al. 2007; Jimenez-Soto et al. 2009; Barden et al. 2013). CagA itself can also bind to integrin α5β1 followed by its internalization into the host cell cytoplasm (Hayashi et al. 2012; Kaplan-Turkoz et al. 2012). *H. pylori* interaction with the host cell plasma membrane also includes direct binding of CagA to externalized membrane phosphatidylserine (PS), an event which is reported to be critical for CagA translocation (Fig. 1) (Murata-Kamiya et al. 2010). A partial crystal structure of N-terminal segments of the protein has been obtained (Hayashi et al. 2012; Kaplan-Turkoz et al. 2012), however, the entire C-terminal part of CagA is not yet crystallized. This part of the protein contains a number of Glu-Pro-Ile-Tyr-Ala (EPIYA)-sequence motifs which can be classified as EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D motifs, depending on their surrounding sequence (Hayashi et al. 2013). In *H. pylori* strains derived from Western countries, single EPIYA-A and EPIYA-B motifs have been reported, typically followed by one to four copies of EPIYA-C, whereas the combination of EPIYA-A and EPIYA-B with single EPIYA-D motifs has been predominantly identified in *H. pylori* isolates isolated in East-Asia (Xia et al. 2009). Strains with higher number of EPIYA-C motifs or presence of EPIYA-D have been associated with an increased risk for the development of gastric cancer (Argent et al. 2004; Jones et al. 2009; Li et al. 2017). However, the situation is not that straightforward. For instance, simultaneous infection with strains expressing diverse CagA EPIYA characteristics have been observed in adult patients (Panayotopoulou et al. 2010) and strains isolated from children do not exhibit multiple EPIYA-C motifs (Sgouras et al. 2009), suggesting that potential increments in the number of repeating EPIYA motifs in CagA occur throughout adulthood. Once intracellular, tyrosine moieties of the EPIYA motifs have been shown to be hierarchically phosphorylated by c-Src and c-Abl family host kinases (Mueller et al. 2012), thereby derailing the host cell function, effectively acting as a molecular "Trojan horse" (Covacci and Rappuoli 2000). How this deregulates downstream signaling processes was summarized in detail in other review articles (Backert et al. 2010; Senda and Hatakeyama 2016; Hatakeyama 2017; Berge and Terradot 2017; Tegtmeyer et al. 2017a). More specifically, a surprisingly high number of over 25 host cell factors have been reported to interact with CagA, in a manner that may or may not depend on EPIYA-phosphorylation, thereby suggesting that CagA can operate as a molecular master key (Backert et al. 2010). A number of key intracellular signaling pathways can be affected, relating to apoptosis and cell cycle proliferation, inflammatory response, cell motility and elongation, intercellular junction integrity or p53-inhibition (Backert et al. 2010; Hatakeyama 2017). Notable interacting targets of the "promiscuous" CagA protein have been identified in a phosphorylation-dependent manner for the SHP-2 phosphatase (Higashi et al. 2002) and in a phosphorylation-independent manner for the tight junction proteins JAM and ZO-1 (Amieva et al. 2003; Krueger et al. 2007), E-cadherin (Murata-Kamiya et al. 2007; Oliveira et al. 2009) and PAR-1 (Hayashi et al. 2012).

A more recent, holistic approach proposed that in order for *H. pylori* to control key host cell signal transduction functions, it injects the CagA protein which functions as a kinase pathway deregulator of a variety of serine/threonine and tyrosine kinases (Tegtmeyer et al. 2017a). These molecules are involved as both receptor- or non-receptor-mediated signaling elements; therefore, CagA seems to be able to manipulate a selection of fundamental cell processes such as adhesion, polarity, proliferation and motility, receptor mediated endocytosis, cytoskeletal rearrangements, apoptosis, inflammation, and cell cycle progression (Fig. 1) (Tegtmeyer et al. 2017a). CagA can accomplish such diverse strategies by activating or deactivating key kinase-dependent pathways. For instance, the Abl

kinase was specifically reported to be activated by CagA (Poppe et al. 2007; Tammer et al. 2007), and so were the carboxy-terminal Src kinase (Csk) (Selbach et al. 2003; Tsutsumi et al. 2003; Selbach et al. 2009), the phosphatidylinositide 3-kinase (PI3K)/Akt pathway (Suzuki et al. 2009; Selbach et al. 2009; Wei et al. 2010; Zhang et al. 2015), the glycogen synthase kinase 3 (GSK-3) (Lee et al. 2014), the Janus kinase (JAK), a family of intracellular, non-receptor tyrosine kinases (Bauer et al. 2012), the Focal adhesion kinase (FAK) (Tegtmeyer et al. 2011), the atypical Protein Kinase C (aPKC) associated with junctional and polarity defects (Saadat et al. 2007; Zeaiter et al. 2008) and MAP kinases. On the other hand, CagA-dependent inactivation has been described for Src kinases (Selbach et al. 2003; Tsutsumi et al. 2003), the partitioning-defective Par1 kinase (Saadat et al. 2007) and the protein kinase C-related kinase 2 (PRK2) (Mishra et al. 2015). Further to GSK-3 targeting, translocated CagA has been suggested to induce epithelial mesenchymal transition (EMT) through EPIYA phosphorylation-dependent up-regulation of metalloprotease MMP-3 (Sougleri et al. 2016). In a phosphorylation-independent manner, translocated CagA has been demonstrated to promote survival of the infected epithelial cells by subverting pro-apoptotic signaling, leading to CagA-dependent p53 degradation (Tsang et al. 2010; Wei et al. 2015, 2010; Buti et al. 2011). Taken together, the CagA protein, following its endocytic translocation, can interact with a number of cellular elements, thus interfering with multiple cell functions and thereby exhibiting a versatile role in *H. pylori* pathogenesis. The elucidation of the exact molecular mechanisms and signaling of these interactions will benefit from structural studies of respective complexes involving full length CagA protein.

The application of animal models of *H. pylori* infection have further highlighted the important role that CagA may play in pathogenesis, as introduction of CagA-positive *H. pylori* into Mongolian gerbils has shown to induce gastric dysplasia and adenocarcinoma, through β-catenin activation and its nuclear accumulation, following CagA translocation (Franco et al. 2005). Further evidence on CagA tumorigenicity was provided following transgenic expression of CagA in C57BL/6J mice, under the control of the β subunit gene promoter of mouse H^+/K^+-ATPase, which resulted in abnormal proliferation of gastric epithelial and hematopoietic cells, thus contributing to the development of gastrointestinal carcinomas and leukemias/lymphomas, in a tyrosine phosphorylation-dependent manner (Ohnishi et al. 2008). Similar observations of the activation of pathways related to oncogenic potential were further supported by other transgenic model systems, including a model using *Drosophila* (Wandler and Guillemin 2012) and another with zebrafish (Neal et al. 2013).

Despite the plethora of reports describing the molecular mechanisms by which CagA can contribute to the bacterial pathogenesis, no clinical recommendations exist with regards to CagA subtyping in the management of patients (Malfertheiner et al. 2017; Chey et al. 2017), although CagA antibodies, which remain positive for a very long period of time, have been suggested to allow detection of *H. pylori* infection in gastric cancer patients when other tests are negative (Malfertheiner et al. 2017). Recent evidence provides further intriguing clues on the complex biology of CagA with regards to its clinical importance, as CagA translocation within gastric epithelial cells has been shown to be dependent on the levels of bacterial hydrogen metabolism. Clinical strains isolated from cancer patients seem to harbor significantly higher hydrogenase activity compared to those derived from patients with gastritis, thereby proposing an association between *H. pylori* hydrogenase activity and gastric carcinogenesis in humans (Wang et al. 2016). Finally, with regards to a role of CagA in pathogenicity, recent evidence suggests that variation in *cagA* gene copy numbers may serve as a novel mechanism by which *H. pylori* can modulate gastric disease development: a considerable proportion of *H. pylori* clinical strains harbor multiple *cagA* copies, which can be differentially associated with gastric disease (Jang et al. 2017). In summary, CagA will continue to intrigue by its mechanistic versatility and fascinating complexity of the evolutionary advantage it may confer to *H. pylori* pathogenesis.

4 *H. pylori* Secretes the Serine Protease HtrA to Shape the Epithelial Barrier

Depolarization of the epithelium represents a hallmark of *H. pylori*-induced gastric carcinogenesis and involves manifold complex pathogen-host interactions that have been summarized in several other review articles (Posselt et al. 2013; Wroblewski and Peek 2007; Hatakeyama 2008). The investigation of bacterial-derived proteases implicated in the disruption of the epithelial barrier function is a relatively new field of research. *H. pylori* expresses HtrA, a protein with dual function acting as a chaperone and a serine protease, which is localized in the periplasm, but is also secreted into the environment (Bumann et al. 2002; Lower et al. 2008). The extracellular localization of HtrA allows a direct interaction with host cell surface molecules. In fact, E-cadherin exposed on gastric epithelial cells was identified as the first substrate for HtrA, that has severe consequences on the epithelial integrity (Hoy et al. 2010).

E-cadherin represents an important cell adhesion molecule, which is essential for the establishment and maintenance of an intact, polarized epithelium. Alterations of E-cadherin function, either through loss-of-function mutations, epigenetically down-regulated gene expression or by protein cleavage, were identified as important steps in gastric carcinogenesis (Liu and Chu 2014; Carneiro et al. 2012). The finding of HtrA-mediated E-cadherin cleavage unravels a novel mechanism in the pathogenesis of *H. pylori* (Wessler and Backert 2017). For a long time, it was suggested that *H. pylori* initiates bacterial pathogenesis via adherence at the apical domain of the epithelium, where it translocates CagA into the cytoplasm. The observation that basolaterally exposed integrin β1 serves as a receptor for the T4SS (Kwok et al. 2007) resulted in the conclusion that *H. pylori* must open intercellular adhesion complexes, which are mainly composed of tight junctions at the transition of the apical to basolateral membrane domains and the subjacent E-cadherin-mediated adherens junctions prior to contact integrin β1 (Fig. 1). Hence, the finding that HtrA cleaves-off the ectodomain of E-cadherin uncovered an elegant mechanism by which *H. pylori* can disrupt intercellular adhesions to open the intercellular space for transmigration (Hoy et al. 2010). Consequently, HtrA-dependent E-cadherin shedding strongly enhances CagA delivery into infected host cells via integrin β1 (Tegtmeyer et al. 2017b). Additional substrates for *H. pylori* HtrA are the extracellular matrix protein fibronectin (Hoy et al. 2010) and the tight junction proteins occludin and claudin-8 (Tegtmeyer et al. 2017b). While the HtrA/E-cadherin interaction is intensively investigated (Schmidt et al. 2016a, b), HtrA-induced cleavage of fibronectin, occludin and claudin-8 needs to be examined in more detail.

H. pylori expresses HtrA ubiquitously and this protease is highly stable under extreme conditions such as high salt concentration, low pH or extreme temperature (Hoy et al. 2013). Until now, *htrA*-negative *H. pylori* isolates have not yet been described as experimental Δ*htrA* knockout mutants are lethal, underlining that the expression of HtrA is essential for bacterial survival (Tegtmeyer et al. 2016; Salama et al. 2004). These observations led to the development of potent HtrA inhibitors in the form of small molecules as well as substrate-derived peptidic inhibitors. The first described small molecule able to inhibit *Helicobacter* HtrA was developed with help of a computational homology model. This *H. pylori* HtrA inhibitor (HHI) efficiently blocked E-cadherin shedding and subsequent bacterial transmigration across a polarized epithelial monolayer (Hoy et al. 2010). Motivated by these results, a large collection of small molecule inhibitors were developed and tested on HtrA activity and *H. pylori*/epithelium interaction (Lower et al. 2011; Geppert et al. 2011; Klenner et al. 2012; Perna et al. 2014, 2015). Through the analysis of the preferred HtrA signature sites in the E-cadherin molecule, an alternative, substrate-derived peptide inhibitor was also found that selectively binds and inhibits HtrA resulting in blocked transmigration of *H. pylori* (Schmidt

et al. 2016b). These studies reveal that pharmacological inhibition of *H. pylori* HtrA can represent a new option in the treatment of *H. pylori* infections.

5 *H. pylori* VacA, GGT and CGT Are Involved in Immune Suppression and Evasion

Previous work identified the *H. pylori* factors VacA, GGT and CGT, which despite a profound effect on gastric epithelial cells, seem to be able to act as immune modulators that impair the activation and proliferation of a variety of immune cells, including T cells, suggesting important roles in immune suppression and evasion (Fig. 2).

6 Vacuolating Cytotoxin A (VacA)

The vacuolating activity associated with *H. pylori* infection of epithelial cells (Leunk et al. 1988) remained controversial with relation to its relevance to pathogenesis, until a protein was purified that seemed responsible for this activity (Cover and Blaser 1992). This VacA was genetically characterized with reference to its pathological significance (Cover et al. 1994; Schmitt and Haas 1994; Telford et al. 1994; Phadnis et al. 1994). Amongst all the *Helicobacter* species known, intact VacA protein with activity associated to gastritis is only present in *H. pylori* and *H. cetorum*, the latter being isolated from marine mammals, potentially suggesting an evolutionary significance (Foegeding et al. 2016).

Early studies have indicated that VacA has a capacity to form anion-selective channels (Czajkowsky et al. 1999; Tombola et al. 1999; Iwamoto et al. 1999), so that VacA was classified as a pore-forming toxin, with vacuolating activity in cell culture assays (Vinion-Dubiel et al. 1999; McClain et al. 2003). Oligomerization into single (hexamers or heptamers) or double layered structures (12-mers or 14-mers) seems to be required for VacA activity, although VacA is believed to initially interact with the plasma membrane of host cells as a monomer, after which it oligomerizes to form a membrane channel (de Bernard et al. 1995; McClain et al. 2000). Phylogenetic analysis of *H. pylori* clinical strains has revealed the existence of several distinct groups of *vacA* alleles (Gangwer et al. 2010). Three main regions of diversity in *vacA* sequences have been recognized, namely the signal sequence region (s-region), the intermediate region (i-region) and middle region (m-region). These result in *vacA* alleles containing multiple combinations of s-, i- and m-region types, relating to variable vacuolating activity (Atherton et al. 1995; Letley and Atherton 2000; McClain et al. 2001; Letley et al. 2003; Rhead et al. 2007), and linked to a potentially higher relative risk for development of gastric cancer or peptic ulcer disease (Figueiredo et al. 2002; Cover 2016).

VacA activity on epithelial cell culture systems have revealed a multitude of effects, varying from endosomal alterations of intraluminal pH (Ricci et al. 1997; Morbiato et al. 2001) and disruption of endocytic compartment trafficking (Satin et al. 1997; Molinari et al. 1998b; Tan et al. 2011), induction of autophagy (Terebiznik et al. 2009; Yahiro et al. 2012) and enhancement of mitochondrial dysfunction, which can result either from its pore-forming ability (Willhite and Blanke 2004) or through the activation of pro-apoptotic factors (Yamasaki et al. 2006). Moreover, VacA activity has been shown to cause increased epithelial barrier alterations through augmented plasma membrane permeability to the extracellular space (Tombola et al. 2001; Debellis et al. 2001), the formation of VacA channels in the plasma membrane (Iwamoto et al. 1999; Tombola et al. 1999) and by increasing paracellular permeability (Papini et al. 1998; Pelicic et al. 1999; Amieva et al. 2003). Finally, VacA-induced effects on epithelial cells include extensive alterations in cell signaling, related to MAP kinase p38 (Nakayama et al. 2004; Hisatsune et al. 2007) and ERK1/2 activation (Nakayama et al. 2004), VEGF upregulation (Caputo et al. 2003) and β-catenin nuclear localization (Nakayama et al. 2009) with subsequent reduction in the expression of pro-survival factors (Matsumoto et al. 2011). Moreover, it has been shown to inhibit gastric acid secretion from parietal cells (Kobayashi et al. 1996; Wang et al. 2008).

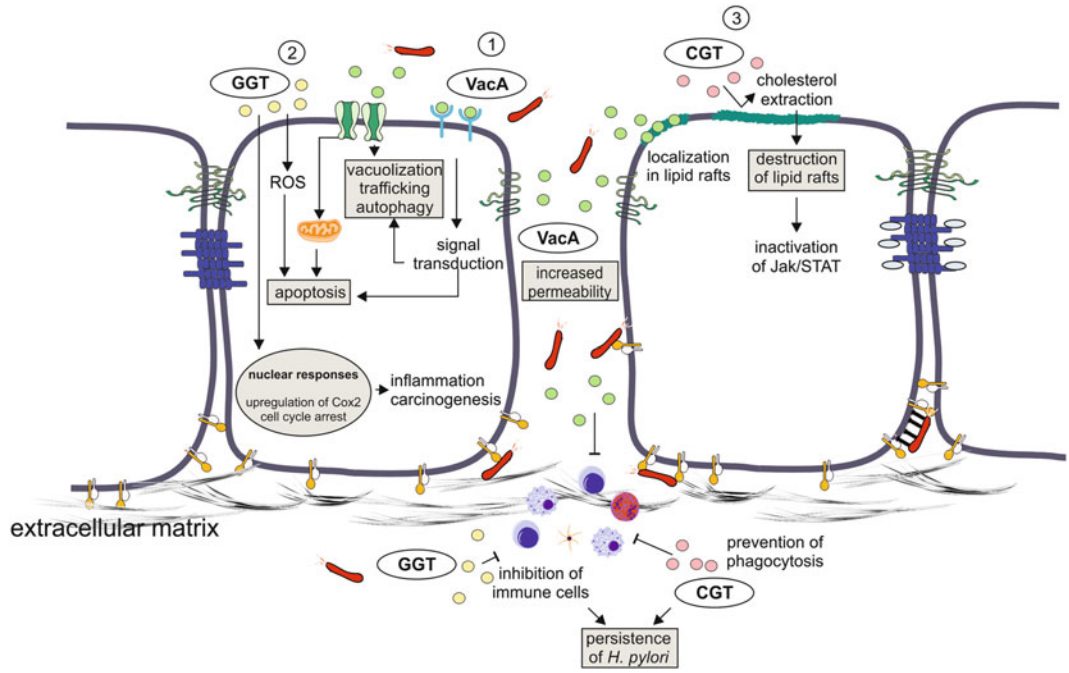

Fig. 2 An interplay of soluble *H. pylori* factors in bacterial persistence. Pleiotropic VacA is secreted by *H. pylori* and can form anion-selective channels leading to extensive vacuolization, changes in compartment trafficking, apoptosis, and autophagy. Vacualization also results in an increased permeability of the epithelial barrier through the disruption of TJs. Further, VacA-induced effects on epithelial cells lead to extensive alterations in cell signaling related to cell survival and cell death, in response to binding to cell surface receptor or localization within lipid rafts. Subsequently, VacA can inhibit the function and proliferation of T cells, B cells, eosinophils, macrophages, dendritic cells and neutrophils (*1*). Soluble GGT is responsible for the conversion of glutamine and glutathione into glutamate. This damages epithelial cells through the production of ammonia and generation of ROS, inducing a cell-cycle arrest and upregulating COX-2 in gastric epithelial cells. Similar to VacA, GGT has been described to inhibit immune cell function. Therefore, inducing *H. pylori* persistence (*2*). *H. pylori* depletes cholesterol from the cell membrane and incorporates it into the bacterial membrane where it is glycosylated by CGT. This results in a destruction of lipid rafts. It further inactivates the JAK/STAT1 signal transduction pathways in primary gastric cells. CGT has also been associated with antiphagocytosis and T-cell inhibition (*3*)

With regards to its ability to act as an immunomodulator, VacA has been demonstrated to inhibit the function and proliferation of a variety of immune cells, such as T cells (Gebert et al. 2003; Utsch and Haas 2016), B cells (Torres et al. 2007), eosinophils (Kim et al. 2007, 2010a), macrophages (Allen et al. 2000; Zheng and Jones 2003), dendritic cells (Kim et al. 2011; Oertli et al. 2013; Djekic and Muller 2016) and neutrophils. Furthermore, the immunomodulatory activity of VacA has been demonstrated in *in vivo* experimental infection models (Oertli et al. 2013; Engler et al. 2014; Kyburz et al. 2017). Such diverse immune functions of VacA accentuate its significant role in the tempering of an immune response in order to facilitate colonization of the gastric epithelium as well as its potential immunomodulatory role on extragastric diseases (Djekic and Muller 2016).

A number of receptors have been proposed for the adhesion of VacA to host cells; however, it remains unclear whether VacA binds to a single abundant, low-affinity receptor or to multiple cell surface components (Foegeding et al. 2016). Candidates include receptor protein tyrosine phosphatase (RPTP) members α and β (Yahiro et al. 1999; Fujikawa et al. 2003; Yahiro et al. 2003, 2004), low-density lipoprotein receptor-related protein-1 (LRP1) (Yahiro et al. 2012), epidermal growth factor receptor (EGFR) (Seto

et al. 1998), heparan sulphate (Utt et al. 2001), sphingomyelin (Gupta et al. 2008; Gupta et al. 2010), glycosphingolipids (Roche et al. 2007), and phospholipids (Molinari et al. 1998a). Of these, only sphingomyelin is suggested to dictate the extent to which VacA binds to the cell surface with subsequent VacA-dependent vacuolation (Foegeding et al. 2016) and sphingomyelin is thought to be the reason for VacA localization within lipid rafts (Geisse et al. 2004; Raghunathan et al. 2018).

In accordance to *in vitro* observations, animal studies have suggested that although,VacA may not be essential for gastric colonization, infection with *H. pylori* strains producing the most active forms of VacA (s1-i1) can induce more severe gastric inflammatory response and extensive metaplasia compared to strains with less active VacA of the s1-i2 or s2-i2 types (Winter et al. 2014). Whether VacA activity is related to gastric carcinogenesis due to impaired tumor surveillance, as a result of its immunomodulatory activity, or due to the augmentation of inflammatory response (Elinav et al. 2013) remains to be clarified – possibly, all three effects may attribute to the pathology.

7 Gamma-Glutamyl Transpeptidase (GGT)

The enzyme GGT catalyzes the transpeptidation and hydrolysis of the gamma-glutamyl group of glutathione and related compounds and is abundant amongst gastric *Helicobacter* species (Rossi et al. 2012). In *H. pylori*, it is synthesized as a proenzyme which is activated through autocatalysis, to form a heterodimer of two subunits of ~40 and 60 kDa, respectively (Boanca et al. 2006). Purified *H. pylori* GGT has a high hydrolyzing activity for conversion of glutamine and glutathione to glutamate with very high affinity for the substrates, indicating a central physiological role of this enzyme in glutamate biosynthesis (Shibayama et al. 2007). However, it has been shown to exhibit a pleiotropic activity both on both gastric epithelial cells and on T-cell mediated immunity. Related to its role in glutamate synthesis, a number of studies have shown that GGT is required for bacterial colonization, since knock-out mutants have exhibited a diminished (McGovern et al. 2001) or even completely abolished (Chevalier et al. 1999) ability to colonize the gastric mucosa in animal models. Furthermore, analysis of clinical strains has suggested that higher GGT activity is associated with peptic ulcer disease while lower GGT activity is more typically observed in strains causing non-ulcer dyspepsia (Gong et al. 2010). Consequently, a damaging effect of GGT on epithelial cells has been associated with the production of ammonia and generation of ROS, leading to caspase-9 and caspase-3 activation and apoptosis (Shibayama et al. 2003, 2007), ATP-depletion and necrosis (Flahou et al. 2011) as well as cell-cycle arrest at G1-S phase (Kim et al. 2010b). Moreover, it was demonstrated that GGT-induced up-regulation of EGF-related peptides and COX-2 in gastric epithelial cells could effectively contribute to the proinflammatory and procarcinogenic effect of *H. pylori* infection (Busiello et al. 2004).

In addition to the effect on epithelial cells, GGT has also been documented to modulate T-cell mediated immunity and thus contributes to immune evasion during infection. More specifically, GGT was identified as the secreted protein responsible for the G1 phase arrest of T cells through disruption of Ras MAPK-dependent signaling (Gerhard et al. 2005), independent of the VacA-dependent T cell proliferation arrest. Collectively, GGT and VacA can inhibit T cell proliferation and differentiation to Th1 and Th17 (Gerhard et al. 2005; Schmees et al. 2007; Beigier-Bompadre et al. 2011). Furthermore, GGT- and VacA-dependent effects on T-cells were suggested to be effected through dendritic cell reprogramming (Oertli et al. 2013), leading to interleukin-10 (IL-10) and IL-18 production and promotion of Treg differentiation that could further suppress Th1 and Th17 effector functions. Such activities exerted by GGT and VacA were associated with an increased protection against allergen-induced asthma, presumably by preventing airway hyper-responsiveness, bronchoalveolar eosinophilia, pulmonary

inflammation and Th2 cytokine production, as was shown in mice tolerized with *H. pylori* extracts applied orally or intraperitoneally (Engler et al. 2014). Depletion of extracellular levels of glutamine by GGT could also result in the impairment of immune functions of the recruited inflammatory cells (Kabisch et al. 2016) and *H. pylori* GGT has been demonstrated to alter T lymphocyte metabolic reprogramming by depriving them from glutamine (Wustner et al. 2017).

8 Cholesterol-α-Glucosyltransferase (CGT)

H. pylori lacks the necessary components for independent sterol synthesis. During infection the bacteria migrate towards a cholesterol gradient and efficiently extract cholesterol from gastric epithelial cell membranes to incorporate glycosylated and non-glycosylated cholesterol into the bacterial membrane (Wunder et al. 2006). The enzyme cholesterol-α-glucosyltransferase (CGT) was identified to glycosylate cholesterol; it is encoded by the *hp0421* gene (Lebrun et al. 2006). The expression of CGT correlates to cholesterol depletion of host membranes, resulting in severe destruction of lipid rafts (Wunder et al. 2006). In initial studies, it was found that incorporation of non-glycosylated cholesterol could enhance phagocytosis by antigen-presenting cells (APCs) and T cell activation, which led to protection against *H. pylori* infections. In contrast, cholesteryl-glucosides abrogated the uptake of *H. pylori* by APCs. Consequently a CGT-negative *H. pylori* deletion mutant was rapidly cleared in a mouse animal model (Wunder et al. 2006), demonstrating that CGT activity can function as a new factor implicated in immune evasion and persistent infection.

The molecular mechanism of CGT-dependent immune evasion is still elusive, but it was indicated that *H. pylori* CGT can induce phagosome maturation arrest, which also involves PI3K activity (Du et al. 2016). In other cell types, such as primary gastric cells, it was proposed that decreased cholesterol levels in host cell membranes caused by *H. pylori* CGT activity not only disrupt lipid rafts, but also prevent IFNγ receptor-mediated signal transduction (Morey et al. 2017). This leads to an inactivation of JAK/STAT1 signal transduction pathways, which creates a micro-niche with lower concentrations of T-cell chemotactic attractants and anti-microbial peptides (human β-defensin 3, hBD3) (Morey et al. 2017). In summary, CGT has emerged as a novel *H. pylori* virulence factor that contributes to gastric carcinogenesis via promoting persistent infections together with T-cell inhibition.

9 Concluding Remarks

H. pylori is one of the most successful pathogens in the world, which colonizes the human gastric mucosa to induce a diverse range of gastric disorders and diseases. Since early human development, *H. pylori* coevolved with the human species through the development of a number of sophisticated strategies, leading to evasion of host surveillance and increased bacterial persistence. In particular, bacterial virulence and pathogenic factors, through their capability to specifically interfere with host cell components, contribute to a highly dynamic and complex pathomechanism. In this review we summarized the function of a number of putative bacterial virulence factors, such as T4SS, CagA, HtrA, VacA, CGT or GGT and examined the mechanisms by which they interfere with the gastric epithelial barrier and immune system. Moreover, these virulence factors seem to interact in synergy, in order to create such conditions of balance between the initial assault, the induction of tolerance and life-long bacterial persistence. These complex associations shaping coevolutionary relationships, between pathogenic *H. pylori* virulence determinants, host factors in inflammatory response genes and environmental factors warrant further careful investigation, necessary for the development of novel pharmacological compounds.

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