Alterations in *Helicobacter pylori* triggered by contact with gastric epithelial cells

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**INTRODUCTION**

*Helicobacter pylori* is highly adapted for colonization of the human stomach, and is found in about half of all humans worldwide (Amieva and El-Omar, 2008; Atherton and Blaser, 2009; Cover and Blanke, 2005; Rieder et al., 2005; Fischer, 2005; Jones et al., 2010). *H. pylori* associates specifically with gastric mucosal tissue in the stomach or the duodenum, but not with intestinal or squamous-type epithelium (Wyatt et al., 1987, 1990; Carrick et al., 1989). The reasons for a specific association between *H. pylori* and gastric epithelial cells are not well understood. One possibility is that *H. pylori* utilizes specific components of gastric mucus or other factors released by gastric epithelial cells as nutritional sources. In addition, *H. pylori* may have a competitive advantage compared to other bacteria in the environment overlying gastric epithelial cells, but may lack this advantage in other sites.

Within the stomach, *H. pylori* can occupy a range of different microenvironments. The bacteria are typically most abundant within the gastric antrum, but can also be found within the corpus. *H. pylori* is found predominantly within the gastric mucus layer (Hazell et al., 1986), but occasionally can be internalized by gastric epithelial cells (Dubois and Boren, 2007); invasion beyond the epithelial layer is considered to be a rare event. Within the gastric mucus layer, the bacteria can be found relatively close to the gastric lumen or deep within gastric glands, and can be either free-swimming (Hazell et al., 1986; Schreiber et al., 2004; Celli et al., 2009) or attached to gastric epithelial cells (Hessey et al., 1990). At any given time, the proportion of adherent *H. pylori* is lower than the proportion of non-adherent organisms. Adherent bacteria localize preferentially to intercellular junctions (Hazell et al., 1986), but also can adhere to non-junctional sites. Relatively little is known about the dynamics of bacterial attachment to gastric epithelial cells. For example, it is not known whether adherent organisms remain attached for short durations and then detach, or whether adherent bacteria remain permanently attached and are eventually shed along with the gastric epithelial cells.

Because *H. pylori* is found in close proximity to gastric epithelial cells, there are numerous opportunities for the bacteria to cause alterations in gastric epithelial cell architecture and function. Many of the changes in gastric epithelial cells caused by *H. pylori* are attributable to the actions of two secreted bacterial proteins, VacA and CagA. VacA is a pore-forming toxin that is secreted by the bacteria through an autotransporter pathway. The cellular alterations caused by VacA include increased permeability of the plasma membrane, changes in endosomal structure and function, changes in mitochondrial membrane permeability, and cell death (Montecucco and de Bernard, 2003; Cover and Blanke, 2005; Rieder et al., 2005; Jones et al., 2010). CagA is an effecter protein that is translated directly from bacteria into host cells through the action of a type IV secretion system (Hatakeyama, 2004; Bourzac and Guillemin, 2005; Rieder et al., 2005; Backert et al., 2010; Fischer, 2011; Tegtmeier et al., 2011; Terradot and Waksman, 2011). Both cagA and genes encoding components of this type IV secretion system are contained within a 40 kb chromosomal region known as...
the cag pathogenicity island (PAI). There is heterogeneity among H. pylori strains, such that strains may contain an intact cag PAI, may contain fragments of this PAI, or may completely lack this region (Olbermann et al., 2010). Upon entry into gastric epithelial cells, CagA interacts with multiple intracellular target proteins and causes a wide array of alterations in cellular signaling, leading to changes in cell shape, increased cellular motility and cellular invasiveness, alterations in monolayer polarity and permeability, and increased cellular proliferation (Hatakeyama, 2004; Bourzac and Guillemín, 2005; Rieder et al., 2005; Backert et al., 2010; Tegtmeier et al., 2011). Because CagA activates signaling pathways associated with carcinogenesis, it has been termed a “bacterial oncoprotein” (Hatakeyama, 2004). The actions of VacA and CagA on epithelial cells have been described in detail in other reviews (Montecucco and de Bernard, 2003; Hatakeyama, 2004; Bourzac and Guillemín, 2005; Cover and Blanke, 2005; Rieder et al., 2005; Backert et al., 2010; Jones et al., 2010; Tegtmeier et al., 2011) and will not be discussed in detail here.

The effects of H. pylori on gastric epithelial cells and the specific bacterial factors that mediate these effects have previously been described in great detail. In contrast, relatively little work has been done to investigate alterations in H. pylori that may be triggered by bacterial contact with human cells. In this article, we review the multiple ways in which contact with gastric epithelial cells causes alterations in H. pylori.

**TRANSCRIPTIONAL REGULATION OF H. PYLORI GENES IN RESPONSE TO BACTERIAL CONTACT WITH GASTRIC EPITHELIAL CELLS**

Several studies have reported that H. pylori adherence to gastric epithelial cells triggers alterations in H. pylori gene transcription (Joyce et al., 2001; van Amsterdam et al., 2003; Kim et al., 2004; Gieseler et al., 2005). These alterations have been detected using a variety of approaches, including transcriptional reporter assays, quantitative real-time PCR (RT-PCR), and array-based hybridization methods.

To identify bacterial genes that are differentially expressed upon bacterial contact with gastric epithelial cells, one study analyzed a plasmid library derived from H. pylori strain 1061, containing random chromosomal fusions to a promoterless cat gene, which allowed chloramphenicol resistance to be used as a marker of gene expression (van Amsterdam et al., 2003). Twenty-one unique clones exhibited increased resistance to chloramphenicol in the presence of HM02 gastric epithelial cells, compared to the level of chloramphenicol resistance in the absence of these cells. Most of the clones were not characterized in detail, but one that exhibited a marked increase in chloramphenicol resistance contained a fusion to vacA (encoding the secreted toxin VacA; van Amsterdam et al., 2003). RT-PCR confirmed that bacterial contact with gastric epithelial cells resulted in increased levels of vacA transcription. These results provide evidence that vacA expression is upregulated upon bacterial contact with gastric epithelial cells.

Effects of cell contact on H. pylori gene transcription have also been assessed by using transcriptional reporter assays to monitor the expression of selected genes (Joyce et al., 2001). The transcriptional reporters were constructed by fusing nine putative promoter regions from the cag PAI with ureB (encoding the B subunit of urease). These reporters were introduced into the hpn locus of an H. pylori strain (C57) in which the endogenous ureB locus had been disrupted. When co-cultured with HEP-2 cells, two of the reporter strains [containing promoters upstream from cagP (cag15) and cagG (cag21)] exhibited increased UreB expression in comparison to when the bacteria were cultured in medium alone (Joyce et al., 2001). The other reporter strains (containing putative promoter regions of seven other cag genes) did not exhibit any significant increase in UreB expression following attachment to HEP-2 cells. A recent report, which describes the use of a high-throughput approach to analyze the transcriptome of H. pylori, verified that a transcriptional start site is present upstream from cagP, but a transcriptional start site was not identified immediately upstream from cagG (Sharma et al., 2010). Instead, it was reported that cagG is transcribed within an operon that originates further upstream (with cagC as the first gene), or as part of a suboperon (with cagF as the first gene) (Sharma et al., 2010). Multiple genes within these operons might be upregulated upon attachment of H. pylori to epithelial cells.

Macroarray hybridization methods have also been used to detect changes in H. pylori gene transcription following bacterial attachment to AGS gastric epithelial cells (Kim et al., 2004). When H. pylori strain 69a was co-cultured with these cells, the transcription of 22 H. pylori genes was upregulated and transcription of 21 genes was downregulated. Regulation of a subset of these genes was confirmed by PCR-based methods. The list of genes confirmed to be regulated included one in the cag PAI (cag3), two encoding outer membrane proteins (omp6 and omp11), and genes encoding proteins involved in chemotaxis and motility (flaA and flgB), transport and binding functions (tonB), metabolic functions (sodB), and transcription- and translation-related functions (Kim et al., 2004).

Another study used quantitative RT-PCR to compare transcription levels of five H. pylori genes following infection of AGS cells with eight different H. pylori strains (Gieseler et al., 2005). Changes in gene expression in individual strains were reproducible, but many results were strain-specific. For example, upon infection of AGS cells, cagA mRNA was upregulated in one strain and downregulated in two other strains (Gieseler et al., 2005). Similar variation was also observed in the transcriptional regulation of katA (a catalase important for oxidative defense), napA (neutrophil activating protein), ureA (a urease subunit), and vacA. Therefore, there may be variation among H. pylori strains in the genes that are differentially expressed following contact with gastric epithelial cells.

Collectively, these reports provide evidence that H. pylori contact with gastric epithelial cells leads to alterations in bacterial gene transcription. Notably, there is very little overlap among the genes that have been identified. Some of the variability may be attributed to variation in study design. Differences in the choice of bacterial strains or cell lines also contribute to the variability. Despite the considerable variability in results, some cohesive elements can be found among these reports. For example, genes encoding virulence factors (including vacA and genes in the cag PAI) have been identified in several studies (Joyce et al., 2001; van Amsterdam et al., 2003; Boonjakuakul et al., 2004, 2005; Kim et al., 2004; Gieseler
et al., 2005; Scott et al., 2007; Castillo et al., 2008). Alterations in host cells induced by these virulence factors are predicted to alter the gastric environment in a manner that is favorable for *H. pylori*.

At present, very little is known about the mechanisms through which contact with epithelial cells leads to alterations in *H. pylori* gene transcription. One possibility is that specific factors (including ions, small molecules, or peptides) are released or secreted by the epithelial cells and sensed by adherent *H. pylori*. Conversely, if specific factors are bound, internalized, or metabolized by the epithelial cells, there may be a reduced concentration of these factors, and the reduced concentration might be sensed by adherent *H. pylori*. A second possibility is that binding of *H. pylori* to components on the surface of host cells might trigger alterations in the bacteria. A third possibility is that epithelial cells might release factors that can be used as nutrients by *H. pylori*, thereby stimulating bacterial metabolism and growth. Although multiple variables, including pH and concentrations of various metals or other ions are known to have an effect on *H. pylori* gene transcription (Merrell et al., 2003a;b; Loh et al., 2007), thus far the factors that stimulate transcriptional alterations in adherent *H. pylori* have not been identified. Similarly, the signaling pathways that may be involved in mediating cell contact-induced alterations in *H. pylori* gene transcription have yet to be identified.

There has been considerable interest in identifying genes that are upregulated during *H. pylori* colonization of mammalian hosts, compared to bacterial growth *in vitro* (Graham et al., 2002; Boonjakuakul et al., 2004, 2005; Scott et al., 2007; Castillo et al., 2008). One approach involved the use of recombination-based *in vivo* expression technology (RIVET) analysis to detect *H. pylori* gene transcription in mice (Castillo et al., 2008). Among the six identified bacterial promoters that were induced in the host compared to *in vitro* conditions, three were predicted to regulate genes with potential roles in host colonization. Specifically, the promoter region upstream of *cagZ* (which likely controls expression of three genes in the *cag* PAI), the promoter predicted to regulate *mobA*, *mobB*, and *mobD* genes (which have roles in horizontal gene transfer of plasmid DNA), and the region upstream of a *vacA* paralog (HP0289, which encodes an autotransporter protein of unknown function) were all found to be upregulated in the host. Analysis of deletion mutant strains revealed that *cagZ*, *mobA*, *mobB*, and *mobD* are important for *H. pylori* colonization of mice (Castillo et al., 2008). Another study analyzed *H. pylori* gene expression during infection of rhesus macaques, and reported that the expression of seven genes, including several from an operon within the *cag* PAI, was increased early in infection of rhesus macaques in comparison to *H. pylori* growth *in vitro* at stationary phase (Boonjakuakul et al., 2005). In contrast, the transcription of many other genes, including five genes from the *cag* PAI, was decreased *in vivo* compared to stationary phase *in vitro* (Boonjakuakul et al., 2005). Although the experiments with animal models provide important insight into the differential expression of *H. pylori* genes *in vivo* and *in vitro*, it is not possible to discern whether the observed changes in gene expression *in vivo* result from bacterial contact with gastric epithelial cells or from bacterial exposure to other environmental conditions.

### ASSEMBLY OF PILI FOLLOWING *H. PYLORI* CONTACT WITH GASTRIC EPITHELIAL CELLS

In addition to the observed alterations in *H. pylori* gene transcription that occur upon bacterial contact with gastric epithelial cells, alterations in bacterial cell morphology also occur. Specifically, extracellular structures termed “pili” are formed, and extend from the surface of the bacteria to the surface of the gastric epithelial cells (Figure 1). Multiple studies have reported that these structures are produced when *H. pylori* is co-cultured with gastric epithelial cells, whereas the structures are produced infrequently when *H. pylori* is cultured in the absence of epithelial cells (Rohde et al., 2003; Kwok et al., 2007; Jimenez-Soto et al., 2009; Shaffer et al., 2011). Initial studies noted that pili were synthesized by a wild-type *H. pylori* strain when in contact with gastric epithelial cells, but not by a mutant strain lacking the *cag* PAI (Rohde et al., 2003). Several individual genes within the *H. pylori* *cag* PAI have been reported to be required for pilus formation (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011). Since the *cag* PAI encodes components of a T4SS that translocates CagA into host cells (Fischer et al., 2001), these pili are considered to be bacterially encoded structures associated with the T4SS, rather than protrusions from the gastric epithelial cell.

Similar pili are features of the T4SSs of several bacterial species, including the *Agrobacterium tumefaciens* VirB/VirD4 system, T4SSs of *Legionella* and *Brucella*, and T4SSs associated with plasmid-encoded conjugation systems (Frost et al., 1986, 1994; Lai and Kado, 1998, 2000; Eisenbrandt et al., 1999; Lai et al., 2000; Aly and Baron, 2007; Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). The *Agrobacterium* VirB/VirD4 system serves as a model for understanding T4SS assembly and architecture (Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). The pili encoded by this system, known as T-pili, are about 10 nm in diameter (Lai and Kado, 2000; Lai et al., 2000; Aly and Baron, 2007; Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009), which allows them to be distinguished from other types of pili (3 nm diameter) and flagella (15 nm diameter). Plasmid-encoded conjugative pili are also 8–12 nm in diameter (Frost et al., 1986, 1994;
VirB5 is required for pilus production (Krall et al., 2002; Yuan et al., 2003). As these proteins have been localized to the pilus in T4SSs of other bacterial species, it is somewhat surprising that none of T4SS components (VirB7, VirB9, and VirB10, respectively) that are present on the sides of the pili in patches or continuous extensions, these proteins may have a role in facilitating bacterial contact with host cells (Yeo et al., 2003; Hwang and Gelvin, 2004; Alvarez-Martinez and Christie, 2009). T-pilus biogenesis requires multiple virB genes, but not virD4 (Pullmer et al., 1996; Lai et al., 2000; Fronzes et al., 2009).

Several Cag proteins have been detected as constituents of H. pylori cag T4SS-associated pili, using immunogold labeling and electron microscopic methods. These include CagY, CagT, CagX, and CagL, as well as the effector protein CagA (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Jimenez-Soto et al., 2009). Immunoelectron microscopy analyses revealed that CagY is present on the sides of the pili in patches or continuous extensions, and it was proposed that CagY represents a pilus sheath (Rohde et al., 2003). CagY is characterized by numerous repeat units; variation in these repeat units may provide a means for evading host immune defenses (Liu et al., 1999; Aras et al., 2003; Delahay et al., 2008). CagT was detected at the base of the pili in one study and along the length of the pili in another study (Rohde et al., 2003; Tanaka et al., 2003). Since CagT, CagX, and CagY are homologs of T4SS components (VirB7, VirB9, and VirB10, respectively) that comprise a core complex spanning the inner and outer membranes in T4SSs of other bacterial species, it is somewhat surprising that these proteins have been localized to the pilus in H. pylori. Each of these proteins has been localized to a H. pylori membrane fraction, and there is evidence that CagT, CagX, and CagY are constituents of a protein complex (Kutter et al., 2008); these observations suggest that these proteins might comprise a T4SS core complex in H. pylori.

Helicobacter pylori CagC exhibits weak homology to VirB2, the major structural component of Agrobacterium pili, and therefore it has been proposed that CagC may serve the same function in the H. pylori cag T4SS (Andrzejewska et al., 2006). Although CagC was detected on the surface of H. pylori (Andrzejewska et al., 2006), this protein has not been definitively localized to H. pylori pili. CagL is proposed to have a role corresponding to the VirB5 minor pilus subunit of Agrobacterium T-pili (Backert et al., 2008). CagL exhibits weak sequence homology to the VirB5 ortholog of Brucella spp., but it lacks any substantial sequence relatedness to Agrobacterium VirB5 (Backert et al., 2008). CagL contains a conserved LQxR motif at the C terminus, which is similar to the motif found in VirB5 of Brucella suis and plasmid-encoded conjugation systems (Backert et al., 2008).

The reported dimensions of H. pylori cag T4SS-associated pili vary considerably among different studies. One study reported that the structures measure about 14 nm in width (Shaffer et al., 2011), which is similar to the diameters of pili in T4SSs of other bacterial species (Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). Another study reported that the structures were either 45 or 70 nm in width, depending on whether or not a sheath was present (Rohde et al., 2003). The difference in reported dimensions among studies is not attributable to a difference in H. pylori strains, since H. pylori strain 26695 was used in all of these studies. Potentially the discrepancy in dimensions is attributable to differences in electron microscopy methods. For example, in some studies, images of the pili were generated using a method in which the structures were coated with carbon, whereas in other studies, images were generated using a method in which the structures were coated with a relatively thinner layer of gold (Rohde et al., 2003; Kwok et al., 2007; Jimenez-Soto et al., 2009; Shaffer et al., 2011). Another factor that may help to account for different results among the studies is that the bacteria were not consistently cultured under the same conditions. Some studies visualized exclusively structures that were present when H. pylori was co-cultured with gastric epithelial cells, whereas other studies also visualized structures that were present when the bacteria were cultured in the absence of epithelial cells.

While multiple lines of evidence indicate that the assembly of H. pylori pili requires genes in the cag PAI (Rohde et al., 2003; Kwok et al., 2007; Shaffer et al., 2011), the composition of these pili remains incompletely characterized and the complete set of genes required for pilus biogenesis has not been defined. Biochemical analysis of these pili has been difficult because the assembly of these structures requires co-culture of H. pylori with gastric epithelial cells; pili are produced infrequently when H. pylori is grown in the absence of gastric epithelial cells (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011). Several individual genes within the H. pylori cag PAI, including cagI, cagL, cagT, cagX, and cagY, are reported to be required for biogenesis of pili (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011), and these genes are also required for CagA translocation into host cells (Fischer et al., 2001; Shaffer et al., 2011). In many cases, the evidence supporting a role of these genes in pilus production was reported as “data not shown” and complemented mutant strains were not analyzed. One study reported that caga/virB11 ATPase was required for pilus formation, and another study reported that it was not required (Tanaka et al., 2003; Kwok et al., 2007). Since at least fourteen genes encoded within the cag PAI are essential for CagA translocation (Fischer et al., 2001), it seems probable that several additional Cag proteins might be required for pilus formation. It is notable that pilus biogenesis requires at least two genes (cagL and cagI) that lack obvious homologs in T4SSs of other bacterial species (Shaffer et al., 2011), and CagL has been detected as a structural component of H. pylori pili (Kwok et al., 2007). This suggests that there are unique features of the H. pylori T4SS compared to T4SSs in other bacteria.

The dimensions of appendages on the surface of bacteria can be regulated by a variety of processes. For example, molecular rulers such as YscP of Yersinia pestis and FliK flagellar protein of Salmonella spp. control the dimensions of T3SS needles and flagella, respectively (Makishima et al., 2001; Journet et al., 2003; Mota et al., 2005). As a clue into the mechanisms by which assembly of H. pylori pili might be regulated, it is of interest that a ΔcagH mutant strain formed pili that were thicker and longer than the pili formed by a wild-type strain (Shaffer et al., 2011). This suggests that CagH has a role in regulating pilus dimensions. The mechanism by which CagH regulates pilus dimensions is not known, but CagH contains a flagellar hook (FigK) domain closely related to
Another hypothesis is that CagA translocation into cells occurs through a pilus-independent process, and the pili merely serve to cue the growth defect of the \textit{cagA} mutant strain, which suggests that cellular alterations induced by CagA may occur in a localized manner.

As a potential mechanism for the observed growth of \textit{H. pylori} on the apical surface of polarized epithelial cells, it has been proposed that the bacteria can derive nutrients from the epithelial cells, and that acquisition of such nutrients permits growth of adherent bacteria. Thus far, acquisition of iron has been studied in greatest detail. One series of experiments showed that although \textit{cagA} mutant bacteria were unable to grow on the apical surface of polarized epithelial cells, the addition of exogenous ferric chloride to the apical medium partially rescued this defect (Tan et al., 2011). Likewise, in comparison to wild-type bacteria, an isogenic \textit{vacA} mutant strain demonstrated decreased microcolony formation on the apical surface of polarized epithelial cells, and addition of iron...
to the apical chamber rescued this defect (Tan et al., 2011). This leads to the hypothesis that CagA and VacA facilitate acquisition of iron from host cells. As a possible mechanism, it was shown that both CagA and VacA contribute to increased basolateral uptake and transcytosis of transferrin as a source of iron (Tan et al., 2011; Figure 2). Mislocalization of the transferrin receptor from the basolateral to the apical surface of polarized cells was also observed. Silencing of transferrin receptor expression resulted in reduced \textit{H. pylori} growth on the apical surface (Tan et al., 2011). The ability of CagA to increase transferrin uptake was dependent on intact CagA EPIYA motifs, which are sites where CagA undergoes tyrosine phosphorylation within host cells. Although VacA is involved in apical mislocalization of the transferrin receptor to sites of bacterial attachment, the addition of exogenous VacA to cells did not stimulate transferrin receptor mislocalization. This suggests that the observed alterations in transferrin receptor localization are dependent on delivery of VacA by intact bacteria.

These studies using polarized epithelial monolayers provide important insights into the process whereby \textit{H. pylori} can obtain iron from host cells, and reveal that CagA and VacA have important roles in this process. It is notable that the addition of iron to the apical medium only partially rescued the ability of a ΔcagA mutant to grow on the apical surface of polarized cells (Tan et al., 2011). This leads to the hypothesis that the bacteria may acquire a variety of other nutrients besides iron from host cells.

CONCLUSION
The surface of gastric epithelial cells represents an important niche for \textit{H. pylori}. The literature discussed in this review, focusing on alterations in \textit{H. pylori} that occur upon bacterial contact with these cells, provides a glimpse of the complex interactions that occur at the bacteria–host cell interface. Alterations in \textit{H. pylori} gene transcription upon bacterial contact with gastric epithelial cells have not yet been investigated in detail, but the finding that transcription of \textit{vacA} and several \textit{cag} PAI genes may be altered provides an initial framework for understanding changes that the bacteria undergo in this environment (Joyce et al., 2001; van Amsterdam et al., 2003; Boonjakuakul et al., 2004, 2005; Kim et al., 2004; Gieseler et al., 2005; Scott et al., 2007; Castillo et al., 2008).

Upon contact with gastric epithelial cells, the bacteria do not only change their transcriptional profile, but also form distinct pilus-like structures. Changes in bacterial transcription and the formation of these pili probably facilitate the delivery of bacterial effector molecules, such as VacA and CagA, into host cells. These effector molecules cause cellular changes that result in an increased availability of iron and other nutrients to the bacteria. Upon uptake of these nutrients, \textit{H. pylori} alters its behavior by replicating and forming microcolonies on the apical cell surface. We speculate that specific components on the surface of gastric epithelial cells (or specific environmental conditions present at the host–pathogen interface) are sensed by \textit{H. pylori}, and that the bacteria accordingly modulate their morphology and ultrastructure to adapt to this environment. Such alterations may optimize the ability of \textit{H. pylori} to proliferate and may promote persistent colonization of the host.

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